Características extraoculares del paciente con retinopatía diabética: arteriosclerosis y capacidad transportadora de oxígeno de la sangre

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Características extraoculares del paciente con retinopatía diabética: arteriosclerosis y capacidad transportadora de oxígeno de la sangre.

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A mi familia y a Toni, los pilares de mi vida

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RESUMEN

CONTEXTO

La diabetes es una enfermedad crónica con complicaciones tardías que clásicamente se han dividido en micro y macroangiopáticas en función de la afectación vascular que comportan. Estos pacientes tienen un riesgo aumentado de morbilidad y mortalidad cardiovascular, sobre todo a expensas de la enfermedad coronaria. Este riesgo aumenta en los pacientes con retinopatía diabética (RD), considerada como un marcador de daño microvascular generalizado en los órganos diana de los pacientes con diabetes mellitus (DM) tipo 2. En la mayoría de los casos, los eventos cardiovasculares que acontecen son producidos por el desarrollo y posterior inestabilidad de la placa de ateroma. Aunque la arterioesclerosis que presentan los pacientes con DM tiene una base común a la de la población no diabética, la hiperglucemia juega un papel determinante en la aceleración y la gravedad del proceso aterogénico, apareciendo de forma más precoz, con una progresión más rápida y una afectación más extensa y periférica. En este sentido, es importante conocer y detectar los cambios precoces que acontecen en la arteriosclerosis subclínica de los pacientes diabéticos. Hasta el momento se conoce que las lesiones más precoces en el desarrollo de la placa de ateroma son el engrosamiento del complejo íntima-media arterial (GIM) y el incremento de la red microvascular de la adventicia arterial, conocida también como vasa vasorum (VV), y que tiene como finalidad el suministro de oxígeno y nutrientes a la parte más externa de la pared vascular. Estímulos como la hipoxia y la isquemia de la pared vascular son capaces de estimular su angiogénesis, de la misma manera que estímulos hipóxicos pueden contribuir, a nivel retiniano, a la neurodegeneración y al daño microvascular que observamos en pacientes diabéticos.

En este sentido, proponemos que la menor capacidad transportadora de oxígeno de la sangre es un factor contribuyente para la aparición y progresión de cambios neuronales y vasculares retinianos, y sugerimos la existencia de fenómenos neurodegenerativos y de grosor retiniano en etapas iniciales de la RD.

Considerando la RD como un factor de riesgo independiente para la enfermedad coronaria incipiente y el accidente cerebrovascular isquémico, planteamos la asociación de la RD con una mayor presencia de enfermedad ateromatosa subclínica carotidea en comparación con los pacientes sin RD, independientemente de otros factores que pueden contribuir a la arteriosclerosis.

Por último, planteamos que la microangiopatía diabética puede afectar a órganos no considerados clásicamente como dianas de esta complicación de la enfermedad. En concreto, planteamos la existencia de microangiopatía que afecta a los vasa vasorum de la pared de la

arteria carótida de pacientes con DM tipo 2. Pensamos que existe una contribución de la enfermedad microvascular carotídea en el proceso arteriosclerótico, y que existe una asociación con la presencia de retinopatía diabética como marcador de alteraciones microangiopáticas en el territorio propuesto.

MÉTODOS

Se diseñó un estudio exploratorio, prospectivo, observacional, de casos y controles, compuesto por una población de sujetos con DM tipo 2 con retinopatía (casos) y sin RD, ni otra manifestación mayor de afectación microvascular diabética (controles). Se seleccionaron pacientes de edades entre 40 y 75 años, estratificados en intervalos de 5 años. Se incluyeron pacientes con DM tipo 2 sin evidencia de enfermedad cardiovascular clínica previa. Desde un punto de vista oftalmológico, se excluyeron aquellos pacientes con errores refractivos o patología ocular concomitante que pudieran dificultar el análisis de datos. Realizamos una exploración oftalmológica completa en todos los pacientes, con valoración del área macular y peripapilar por tomografía de coherencia óptica (OCT). Realizamos retinografías funduscópicas y angiografía fluoresceínica en todos aquellos pacientes con algún grado de RD. Determinamos la presencia o ausencia de edema macular clínicamente significativo y de isquemia retiniana, definida por la presencia de isquemia macular y/o periférica acompañante.

Valoramos la señal de vasa vasorum de la pared de la arteria carótida común en parte de nuestra muestra de pacientes diabéticos y en un grupo de voluntarios sanos. En todos ellos realizamos una ecografía con contraste de microburbujas en un segmento de la pared arterial de la carótida común libre de placa. Para valorar el aumento de la carga arteriosclerótica en pacientes con DM tipo 2 y RD, realizamos ecografía carotidea convencional en la arteria carótida común, bulbo y carótida interna. Además, recogimos las características ecográficas de las placas de ateroma, con determinación de los valores de grosor íntima-media (GIM), media y máximo y media global, en los tres territorios estudiados.

RESULTADOS

Los resultados de nuestro estudio no lograron demostrar una asociación independiente entre las concentraciones de hemoglobina (Hb) y la presencia de RD, pero sí demostraron que las concentraciones bajas de Hb se asocian con formas más avanzadas de RD y con la presencia, hasta ahora no descrita, de isquemia retiniana.

También evidenciamos una correlación significativa entre bajos niveles de hematocrito y eritrocitos y el descenso en la capa de fibras nerviosas de la retina (CFNR) en aquellos pacientes sin RD o con RD incipiente. El estudio de grosor y volumen macular evidenció un engrosamiento mayor en los pacientes diabéticos con RD leve respecto a aquellos sin RD, no observándose diferencias en CFNR entre ambos grupos.

En el estudio del VV carotideo, observamos un aumento de la señal de VV en aquellos pacientes diabéticos con RD respecto a aquellos sin RD, como signo indirecto de un incremento de la angiogénesis en la adventicia de la pared de la arteria carótida. Esta asociación se mantuvo después de ajustar por diferentes factores, incluidos todos los de riesgo cardiovascular. Los pacientes diabéticos respecto a los controles sanos, también mostraron aumento de la señal de VV, incluso en ausencia de RD.

La determinación del GIM y de la carga arteriosclerótica (presencia y número de placas de ateroma) en las arterias carótidas de nuestra población de estudio, demostró que los pacientes con DM tipo 2 con RD presentan una mayor prevalencia de arteriosclerosis subclínica en comparación con los pacientes sin RD. La RD se asoció de forma independiente con la presencia de placas carotídeas.

CONCLUSIONES

La baja capacidad de transporte de oxígeno en la sangre, medida como la concentración de hemoglobina circulante, se asocia a estadios más avanzados de retinopatía diabética y a la presencia de isquemia retiniana.

Bajos niveles de hematocrito y eritrocitos se asocian con cambios neuroretinianos precoces, evidenciados por una pérdida de grosor de CFNR en pacientes sin retinopatía diabética o con retinopatía incipiente. El aumento de grosor y volumen macular en diabéticos con RD leve, es un signo precoz que puede indicar el aumento de la permeabilidad vascular.

Los pacientes diabéticos, y de forma más acentuada los pacientes con retinopatía diabética, muestran un aumento de la angiogénesis de los vasa vasorum de la arteria carótida.

Los pacientes con retinopatía diabética presentan mayor frecuencia de enfermedad ateromatosa subclínica carotidea (presencia y número de placas de ateroma) en comparación con los pacientes sin RD, independientemente de otros factores que pueden contribuir a la arteriosclerosis.

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CONTEXT

La diabetis és una malaltia crònica amb complicacions tardanes que clàssicament s'han dividit en micro i macroangiopáticas en funció de l'afectació vascular que comporten. Aquests pacients tenen un risc augmentat de morbiditat i mortalitat cardiovascular, sobretot a expenses de la malaltia coronària. Aquest risc augmenta en els pacients amb retinopatia diabètica (RD), considerada com un marcador de dany microvascular generalitzat en els òrgans diana dels pacients amb diabetis mellitus (DM) tipus 2. En la majoria dels casos, els esdeveniments cardiovasculars que esdevenen són produïts pel desenvolupament i posterior inestabilitat de la placa d'ateroma. Tot i que la arteriosclerosi que presenten els pacients amb DM té una base comuna a la de la població no diabètica, la hiperglucèmia juga un paper determinant en l'acceleració i la gravetat del procés aterogènic, apareixent de forma més precoç, amb una progressió més ràpida i una afectació més extensa i perifèrica. En aquest sentit, és important conèixer i detectar els canvis precoços que esdevenen en l'arteriosclerosi subclínica dels pacients diabètics. Fins al moment es coneix que les lesions més precoces en el desenvolupament de la placa d'ateroma són l'engrossiment del complex íntima-mitja arterial (GIM) i l'increment de la xarxa microvascular de la adventícia arterial, coneguda també com vasa vasorum (VV) , i que té com a finalitat el subministrament d'oxigen i nutrients a la part més externa de la paret vascular. Estímuls com la hipòxia i la isquèmia de la paret vascular són capaços d'estimular la seva angiogènesi, de la mateixa manera que estímuls hipòxics poden contribuir, a nivell retinià, a la neurodegeneració i al dany microvascular que observem en pacients diabètics.

En aquest sentit, proposem que la menor capacitat transportadora d'oxigen de la sang és un factor contribuent per a l'aparició i progressió de canvis neuronals i vasculars retinians, i suggerim l'existència de fenòmens neurodegeneratius i de gruix retinal en etapes inicials de la RD.

Considerant la RD com un factor de risc independent per a la malaltia coronària incipient i l'accident cerebrovascular isquèmic, plantegem l'associació de la RD amb una major presència de malaltia ateromatosa subclínica carotídia en comparació amb els pacients sense RD, independentment d'altres factors que poden contribuir a l'arteriosclerosi.

Finalment, plantegem que la microangiopatia diabètica pot afectar òrgans no considerats clàssicament com a dianes d'aquesta complicació de la malaltia. En concret, plantegem l'existència d'microangiopatia que afecta els vasa vasorum de la paret de l'artèria caròtida de pacients amb DM tipus 2. Pensem que hi ha una contribució de la malaltia microvascular carotídia en el procés arterioscleròtic, i que hi ha una associació amb la presència de retinopatia diabètica com a marcador d'alteracions microangiopàtiques al territori proposat.

MÈTODES

Es va dissenyar un estudi exploratori, prospectiu, observacional, de casos i controls, compost per una població de subjectes amb DM tipus 2 amb retinopatia (casos) i sense RD, ni una altra manifestació major d'afectació microvascular diabètica (controls). Es van seleccionar pacients d'edats entre 40 i 75 anys, estratificats en intervals de 5 anys. Es van incloure pacients amb DM tipus 2 sense evidència de malaltia cardiovascular clínica prèvia.

Des d'un punt de vista oftalmològic, es van excloure aquells pacients amb errors refractius o patologia ocular concomitant que poguessin dificultar l'anàlisi de dades. Vam realitzar una exploració oftalmològica completa en tots els pacients, amb valoració de l'àrea macular i peripapil·lar per tomografia de coherència òptica (OCT). Realitzem retinografies funduscópicas i angiografia fluoresceínica a tots aquells pacients amb algun grau de RD. Determinem la presència o absència d'edema macular clínicament significatiu i d'isquèmia retiniana, definida per la presència d'isquèmia macular i / o perifèrica acompanyant.

Valorem el senyal de vasa vasorum de la paret de l'artèria caròtida comuna en part de la nostra mostra de pacients diabètics i en un grup de voluntaris sans. En tots ells vam realitzar una ecografia amb contrast de microbombolles en un segment de la paret arterial de la caròtida comuna lliure de placa. Per valorar l'augment de la càrrega arterioscleròtica en pacients amb DM tipus 2 i RD, vam realitzar ecografia carotídia convencional a l'artèria caròtida comú, bulb i caròtida interna. A més, vam recollir les característiques ecogràfiques de les plaques d'ateroma, amb determinació dels valors de gruix íntima-mitja (GIM), mitja i màxim i mitjana global, en els tres territoris estudiats.

RESULTATS

Els resultats del nostre estudi no van aconseguir demostrar una associació independent entre les concentracions d'hemoglobina (Hb) i la presència de RD, però sí van demostrar que les concentracions baixes d'Hb s'associen amb formes més avançades de la RD i amb la presència, fins ara no descrita, d'isquèmia retiniana.

També vam mostrar una correlació significativa entre baixos nivells d'hematòcrit i eritròcits i el descens en la capa de fibres nervioses de la retina (CFNR) en aquells pacients sense RD o amb RD incipient. L'estudi de gruix i volum macular va evidenciar un engrossiment més gran en els pacients diabètics amb RD lleu respecte a aquells sense RD, no observant diferències en CFNR entre els dos grups.

En l'estudi del VV carotidi, observem un augment del senyal de VV en aquells pacients diabètics amb RD que fa a aquells sense RD, com a signe indirecte d'un increment de l'angiogènesi en la adventícia de la paret de l'artèria caròtida. Aquesta associació es va mantenir després d'ajustar per diferents factors, inclosos tots els de risc cardiovascular. Els pacients diabètics respecte als controls sans, també van mostrar augment del senyal de VV,

fins i tot en absència de RD.

La determinació del GIM i de la càrrega arterioscleròtica (presència i nombre de plaques d'ateroma) a les artèries caròtides de la nostra població d'estudi, va demostrar que els pacients amb DM tipus 2 amb RD presenten una major prevalença de arteriosclerosi subclínica en comparació amb els pacients sense RD. La RD es va associar de forma independent amb la presència de plaques carotídies.

CONCLUSIONS

La baixa capacitat de transport d'oxigen a la sang, mesurada com la concentració d'hemoglobina circulant, s'associa a estadis més avançats de retinopatia diabètica ia la presència d'isquèmia retiniana.

Baixos nivells d'hematòcrit i eritròcits s'associen amb canvis neuroretinianos precoços, evidenciats per una pèrdua de gruix de CFNR en pacients sense retinopatia diabètica o amb retinopatia incipient. L'augment de gruix i volum macular en diabètics amb RD lleu, és un signe precoç que pot indicar l'augment de la permeabilitat vascular.

Els pacients diabètics, i de forma més accentuada els pacients amb retinopatia diabètica, mostren un augment de l'angiogènesi dels vasa vasorum de l'artèria caròtida.

Els pacients amb retinopatia diabètica presenten major freqüència de malaltia ateromatosa subclínica carotídia (presència i nombre de plaques d'ateroma) en comparació amb els pacients sense RD, independentment d'altres factors que poden contribuir a l'arteriosclerosi.

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SUMMARY

CONTEXT

Diabetes is a chronic disease with late complications that classically have been divided into micro and macroangiopathic according to the vascular affectation that entail. These patients have an increased risk of cardiovascular morbidity and mortality, particularly at the expense of coronary heart disease. This risk is increased in patients with diabetic retinopathy (DR), which is regarded as a marker of microvascular damage widespread in the target organs of patients with diabetes mellitus (DM) type 2. In most cases, the cardiovascular events that occur are produced by the development and subsequent instability of the atheromatous plaque. Although the atherosclerosis that present the patients with DM has a common base to that of the non-diabetic population, the hyperglycemia plays a determinant role in the acceleration and the gravity of the atherogenic process, appearing in premature form, with a faster progression and a peripheral and more extensive affectation. In this regard, it is important to understand and detect the early changes that occur in the subclinical atherosclerosis in diabetic patients. So far, it is known that the earliest injuries in the development of atheromatous plaque are the thickening of the arterial intima-media (IMT) complex and the increase of the microvascular network of the arterial adventitia, also known as vasa vasorum (VV), which has as its aim the supply of oxygen and nutrients to the outermost part of the vessel wall. Stimuli such as hypoxia and ischemia of the vessel wall are able to stimulate angiogenesis, in the same way that hypoxic stimuli can contribute to retinal level, neurodegeneration and the microvascular damage that we observe in diabetic patients.

In this sense, we suggest the lesser oxygen carrying capacity in blood as a contributing factor for the development and progression of neuronal and retinal vascular changes, and we suggest the existence of neurodegenerative phenomena and retinal thickness at initial stages of the DR.

Considering the DR as an independent risk factor for the incipient coronary artery disease and ischaemic stroke, the association of the DR with a greater presence of subclinical carotid atheromatous disease compared with patients without DR, regardless of other factors that can contribute to atherosclerosis was presented.

Finally, we propose that the diabetic microangiopathy can affect organs not classically considered as targets of this complication of the disease. In particular, we propose the existence of microangiopathy that affects the vasa vasorum of the wall of the carotid artery of patients with DM type 2. We believe that there is a contribution of the carotid microvascular disease in the atherosclerotic process, and that there is an association with the presence of diabetic retinopathy as a marker of macroangiopathic alterations in the proposed territory.

METHODS

An exploratory study, prospective, observational, of cases and controls, composed by a population of subjects with DM type 2 with retinopathy (cases) and without DR was designed, neither another greater demonstration of diabetic microvascular affectation (controls). We selected patients aged between 40 and 75 years, stratified in intervals of 5 years. We included patients with DM type 2 without evidence of prior cardiovascular disease clinic. From an ophthalmological point of view, those patients with refractive errors or concomitant ocular pathology that might hinder the analysis of data were excluded. A complete ophthalmological examination in all patients, with valuation of the macular and peripapillary area by optical coherence tomography (OCT) was carried out. Retinographies and fluorescein angiography in all patients with some degree of DR were carried out. The presence or absence of clinically significant macular edema and retinal ischemia, defined by the presence of macular ischemia and/or peripheral companion were determined.

The signal of vasa vasorum of the wall of the common carotid artery in part of our sample of diabetic patients and in a group of healthy volunteers was valued. For the first time the method used is described, being the first study in the literature that uses this methodology. In all of them an ultrasound with contrast of microbubbles in a segment of the arterial wall of the common carotid plaque-free was carried out. To value the increase of the atherosclerotic load in patients with DM type 2 and DR, a conventional carotid ultrasound in the common carotid artery, bulb and internal carotid was carried out. In addition, the ultrasound features of the atheroma plaques, with determination of the values of intima-media thickness (IMT), average and maximum and average overall, in the three territories surveyed was collected.

RESULTS

The results of our study failed to demonstrate an independent association between concentrations of hemoglobin (Hb) and the presence of DR, but they demonstrated that low concentrations of Hb associate with more advanced forms of DR and with the presence, until now not been described, of retinal ischaemia.

A significant correlation between low levels of hematocrit and erythrocytes and the decline in the retinal nerve fiber layer (RNFL) in those patients without DR or with incipient DR was also evidenced. The study of macular thickness and volume showed a greater thickening in diabetic patients with DR slight with respect to those without DR, not being observed differences in RNFL between both groups.

In the study of the VV carotid, an increase in the signal of VV in those diabetic patients with DR with regard to those without DR, as indirect sign of an increase of the angiogenesis in the adventitia of the wall of the carotid artery was observed. This association was maintained after adjusting for various factors, including all those of cardiovascular risk. The diabetic patients

with regard to the healthy controls, also showed an increase in the signal of VV, even in absence of DR.

The determination of the IMT and of the atherosclerotic load (presence and number of plates of atheroma) in the carotid arteries of our population of study, showed that the patients with DM type 2 with DR present a greater prevalence of subclinical atherosclerosis in comparison with the patients without DR. The DR was independently associated with the presence of carotid plaques.

CONCLUSIONS

The low oxygen carrying capacity in blood, measured as the concentration of circulating hemoglobin, is associated with more advanced stages of diabetic retinopathy and the presence of retinal ischaemia.

Low levels of hematocrit and erythrocytes are associated with early neuroretinal changes, evidenced by a loss of thickness of RFNL in patients without diabetic retinopathy and with incipient retinopathy. The increase in thickness and macular volume in diabetic patients type 2 with mild DR, is an early sign that may indicate an increase in vascular permeability.

Patients with diabetes mellitus type 2, and more accentuated the patients with diabetic retinopathy, show an increase in the angiogenesis of the vasa vasorum of the carotid artery. This allows us to suggest the microcirculation of the arterial adventitia as non-classic target tissue of the microangiopathy in diabetes mellitus type 2. This microangiopathy of the arterial wall would contribute at least in part to the development and progression of atherosclerosis in diabetes.

The patients with diabetic retinopathy present a greater frequency of subclinical carotid atheromatous disease (presence and number of atheroma plaques) compared with patients without DR, regardless of other factors that can contribute to atherosclerosis.

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ÍNDICE

1. PRESENTACIÓN

Esta tesis doctoral se estructura según las directrices de la normativa para la presentación de Tesis Doctorales en formato de artículos, según el Acuerdo núm. 19/2002 de la Universidad de Lérida.

Los estudios realizados en la presente Tesis Doctoral forman parte de una línea de investigación iniciada en el 2010 y dirigida a profundizar en el estudio de las características extraoculares del paciente con retinopatía diabética, concretamente en la arteriosclerosis y la baja capacidad transportadora de oxígeno de la sangre.

Los resultados obtenidos han aportado información relevante en este campo y han sido publicados en forma de 2 artículos en revistas de impacto internacional, un tercer artículo se ha enviado a una revista de alto impacto y un cuarto artículo está pendiente de envío.

Título: Microangiopathy of large artery wall: A neglected complication of diabetes mellitus
Autores: Arcidiacono MV, Traveset A, Rubinat E, Ortega E, Betriu A, Hernández M, Fernández E, Mauricio D.
Revista: Atherosclerosis, 2013; 228:142-7
Factor de impacto: 3.994 (Cuartil 1)

Título: Type 2 diabetes-associated carotid plaque burden is increased in patients with retinopathy compared to those without retinopathy.
Autores: Alonso N, Traveset A, Rubinat E, Ortega E, Alcubierre N, Sanahuja J, Hernández M, Betriu A, Jurjo C, Fernández E, Mauricio D.
Revista: Cardiovasc Diabetol. 2015;14:33.
Factor de impacto: 4.015 (Cuartil 1)

Título: Lower hemoglobin concentration is associated with retinal ischemia and the severity of diabetic retinopathy in type 2 diabetes
Autores: Traveset A, Rubinat E, Ortega E, Alcubierre N, Vazquez B, Hernández M, Jurjo C, Espinet R, Ezpeleta JA, Mauricio D
Revista: enviado a la revista British journal of ophthalmology
Factor de impacto: 2.809 (Cuartil 1)

Título: Early changes in retinal thickness and its association with hematologic factors in type 2 diabetes mellitus.
Autores: Traveset A, Rubinat E, Alcubierre N, Vazquez B, Jurjo C, Espinet R, Sanchez C, Muniesa MJ, Huerva V, Mauricio D
Revista: pendiente de envío

Como información adicional, mencionar que la doctoranda ha formado parte del equipo de investigación de los siguientes artículos, centrados también en la pared arterial y la diabetes mellitus:

- Rubinat E, Ortega E, Traveset A, Arcidiacono MV, Alonso N, Betriu A, Granado-Casas M, Hernández M, Soldevila J, Puig-Domingo M, Jurjo C, Fernández E, Mauricio D. Microangiopathy of common carotid vasa vasorum in type 1 diabetes mellitus. Atherosclerosis, 2015; 241:334-8
  Factor de impacto: 3.994

- Alcubierre N, Valls J, Rubinat E, Cao G, Esquerda A, Traveset A, Granado-Casas M, Jurjo C, Mauricio D. Vitamin D Deficiency Is Associated with the Presence and Severity of Diabetic Retinopathy in Type 2 Diabetes Mellitus. J Diabetes Res. 2015; 2015:374178.
  Factor de impacto: 3.536

2. INTRODUCCIÓN

La diabetes mellitus (DM) es la enfermedad metabólica más frecuente en la población mundial. Está ocasionada por un déficit en la secreción de insulina y/o por un defecto en la acción de la misma, lo que produce un trastorno en el metabolismo de los hidratos de carbono con aumento de la concentración de glucosa en sangre y en los tejidos intersticiales. También el metabolismo lipídico y proteico pueden encontrarse alterados. La DM tipo 2 es el tipo de diabetes más prevalente. Se produce por una resistencia a la acción de la insulina, generalmente asociada a la obesidad, a la que se suma una inadecuada respuesta secretora de la misma hormona.

La diabetes es una enfermedad que puede producir complicaciones de forma aguda o crónica. Entre sus complicaciones tardías, se distinguen clásicamente dos tipos principales por la afectación vascular que comportan, las microangiopáticas (retinopatía, nefropatía y neuropatía), y las macroangiopáticas (afectación de grandes vasos)[1]. Estas últimas son las que condicionan la aparición de eventos cardiovasculares según el territorio afectado: enfermedad vascular cerebral, cardiopatía isquémica (angina o infarto), y enfermedad vascular periférica de extremidades inferiores (claudicación, isquemia).

2.1. Complicaciones macroangiopáticas de la DM

Las complicaciones macroangiopáticas constituyen la causa más frecuente de muerte en el paciente con DM, siendo responsables del 65-80% de los fallecimientos de estos pacientes. La afectación macrovascular con mayor morbimortalidad es la de las arterias coronarias, las arterias cerebrales y las arterias de miembros inferiores. La DM no sólo es un factor de riesgo independiente y potente para el desarrollo de arteriosclerosis, sino que, además, cuando los pacientes diabéticos sufren eventos macrovasculares, su gravedad es mayor y su pronóstico peor que en los pacientes no diabéticos.

2.2. Complicaciones microangiopáticas de la DM

Las alteraciones en la microcirculación del paciente diabético pueden alterar la perfusión de algunos órganos, afectando particularmente a aquellos que dependen de dicha red microvascular, tales como la retina, el riñón y el sistema nervioso periférico. Los problemas clínicos asociados a estos cambios –retinopatía, nefropatía y neuropatía- conducen a una gran carga de morbilidad en los pacientes con DM tipo 2.

2.3. Retinopatía diabética

La retinopatía diabética (RD) es la complicación microvascular crónica más frecuente de la diabetes, y se ha convertido en la principal causa de déficit visual y ceguera en adultos en edad laboral en todo el mundo[2]. A nivel global, se ha estimado que alrededor del 30% de las personas con DM tienen RD[3].

2.3.1. Clasificación de la RD

Según la Clasificación clínica Internacional de la RD y el Edema Macular[4] (GDRPC: Global Diabetic Retinopathy Project Group), los pacientes diabéticos pueden clasificarse en función de la gravedad de los cambios microvasculares retinianos, y de la presencia o ausencia de neovascularización retiniana. Los procesos patológicos asociados con la progresión de la RD incluyen la formación de microaneurismas, el incremento de la permeabilidad vascular, la aparición de hemorragias retinianas, de exudados lipídicos y de fenómenos oclusivos vasculares.

Clasificación Clínica Internacional de la RD[4]

Sin RD aparente: Ausencia de microaneurismas (μA)
RD no proliferativa (RDNP) leve: Sólo μA
RDNP moderada: μA asociado a menos de 20 hemorragias intrarretinianas en cada uno de los 4 cuadrantes, exudados duros, exudados algodonosos, arrosariamiento venoso en 1 sólo cuadrante
RDNP grave: μA junto a uno de los siguientes hallazgos: – Hemorragias intrarretinianas graves (>20) en cada uno de los 4C. – Arrosariamiento venoso en ≥2 C. – Anomalías microvasculares intrarretinianas (AMIR) en ≥1 C
RDNP muy grave: μA junto al menos dos de los hallazgos anteriores.
RD proliferativa (RDP): Neovasos (NV) y/o hemorragia prerretiniana o hemovítreo

Los microaneurismas capilares retinianos suelen ser el primer signo visible de la RD. Pueden ser detectados clínicamente por medio de la oftalmoscopia, y son el sello identificativo de la RDNP.

A medida que aumenta la formación de microaneurismas, se produce una hiperpermeabilidad vascular de los capilares retinianos, lo que causa la aparición de edema

retiniano, normalmente en el área macular. Su evaluación suele realizarse por separado del estadío de la RD, puesto que puede tener un curso independiente.

El diagnóstico del edema macular diabético (EMD) tiene actualmente como referencia obligada el estudio ETDRS[5]. Según este grupo, se define como edema macular clínicamente significativo (EMCS) si existe la presencia de cualquiera de las siguientes condiciones:

- Engrosamiento retiniano dentro de las 500 micras centrales contando desde el centro de la fóvea.
- Presencia de exudados duros dentro de las 500 micras centrales si se asocian a engrosamiento de la retina adyacente; pero no los exudados duros que restan tras desaparecer el engrosamiento retiniano.
- Presencia de zonas de engrosamiento de retina del tamaño de un diámetro papilar o mayor; si alguna parte del mismo está dentro de un diámetro papilar, contando desde el centro de la fóvea.

A pesar de que ésta es la clasificación del EMD más aceptada, han existido otras, tales como la clasificación basada en la angiografía fluoresceínica o en la tomografia de coherencia óptica (OCT). La OCT es una prueba complementaria que ofrece imágenes tridimensionales, de alta resolución y con cortes transversales del área macular y peripapilar. Esta técnica aporta medidas precisas y reproducibles del grosor retiniano. En el EMD nos permite monitorizar la progresión y la respuesta al tratamiento. A nivel del nervio óptico, la OCT nos permite estudiar y cuantificar la capa de fibras nerviosas de la retina.

En etapas avanzadas de la RD se produce una obliteración de los capilares retinianos, que se traduce clínicamente como un aumento de hemorragias, anomalías venosas y anomalías microvasculares intrarretinianas. El cierre capilar condiciona la aparición de isquemia retiniana, que promueve la formación de neovasos en la retina y en la superficie posterior vítrea. La aparición de neovascularización retiniana anuncia un cambio crítico en la progresión de la RD. La angiografía con fluoresceína es una técnica de imagen de gran utilidad para evidenciar la presencia de isquemia retiniana, tanto a nivel periférico como macular, y para identificar los neovasos retinianos.

2.3.2. Patogenia

Aunque la causa exacta de la RD es aún desconocida, la hipótesis más probable es que el estado de hiperglucemia mantenida sea el principal responsable. Junto con el aumento de la concentración de glucosa en sangre, existen otros factores de riesgo sistémico y factores genéticos que pueden también contribuir a su desarrollo.

La duración y el tipo de diabetes constituyen los factores de riesgo más importantes para la aparición y la progresión de la RD, de forma que después de 20 años de evolución de la

enfermedad el 95% de los pacientes diabéticos tipo 1 y el 60% de los pacientes diabéticos tipo 2 presentan RD en alguno de sus grados[6]. Este porcentaje es diferente cuando nos referimos a prevalencia de RDP, de manera que, tras 20 años de enfermedad, hasta el 50% de los pacientes diabéticos tipo 1 pueden presentar formas avanzadas de RD, frente a un 10-15% de los tipo 2. La prevalencia de EMD es, sin embargo, mayor en sujetos diabéticos tipo 2, sobre todo en aquellos que reciben insulina como tratamiento[7,8].

El *mal control de la glucemi*a ha demostrado también una clara asociación con la presencia y progresión de la RD y del EMCS en ambos tipos de diabetes. Esta asociación aumenta de manera exponencial a la concentración de hemoglobina glucosilada. Existen estudios que demuestran como el tratamiento optimizado con insulina retrasa la aparición y progresión de todas las complicaciones microvasculares (retinopatía, nefropatía y neuropatía)[9-12].

La *hipertensión arterial* es más prevalente en los pacientes diabéticos y tiene un efecto deletéreo sobre las complicaciones diabéticas. En el estudio UKPDS (United Kingdom Prospective Diabetes Study)[13], estudio prospectivo que examinó además el efecto del tratamiento de la hipertensión arterial (HTA) en la RD, se demostró que la reducción de la presión arterial diastólica por debajo de 85 mmHg y de la presión arterial sistólica por debajo de 150 mmHg proporcionaba una reducción del 34% en el riesgo de progresión de la RD en la diabetes tipo 2.

La *pubertad y el embarazo* pueden acelerar el desarrollo de la RD debido, probablemente, entre otros factores, a los cambios hormonales acompañantes.

Algunos estudios han encontrado una relación positiva ente las *concentraciones elevadas de lípidos séricos* y el desarrollo de exudados duros y la progresión de la RD. Evidencias posteriores han demostrado el beneficio del tratamiento de la dislipemia en la mejora de la progresión de la RD y del edema macular en estos pacientes[14,15].

La presencia de *microalbuminuria* se ha asociado también con la aparición de RD y, además, con el riesgo aumentado de desarrollar nefropatía y complicaciones cardiovasculares en pacientes diabéticos. La RD grave puede ser un indicador de enfermedad renal mientras que la nefropatía y el tratamiento de la misma pueden afectar a la progresión de la RD[16-18].

La *anemia* y los *factores genéticos* también han sido investigados, y han sido relacionados con el desarrollo y progresión de la RD. Las concentraciones bajas de hemoglobina (Hb) se consideran como un factor de riesgo independiente para el desarrollo de RD proliferativa y de pérdida visual grave[19]. Otros estudios han corroborado este hallazgo, y han demostrado una mejoría en el estadio de la RD con la corrección de la anemia[20,21-23].

Los mecanismos fisiopatológicos responsables de la RD van conociéndose cada vez más. Se cree que el daño endotelial de la microcirculación retiniana es el principal responsable del desarrollo de la microangiopatía. Y es la hiperglucemia como factor fundamental, aunque también los otros factores mencionados anteriormente, la que a través de cambios a nivel bioquímico, hematológico, anatómico y fisiológico producen un daño endotelial vascular y sobre las neuronas retinianas. Las lesiones que observamos clínicamente en estos pacientes son secundarias a esta microangiopatía y se traducen en un aumento de la permeabilidad vascular en forma de edema, y en fenómenos oclusivos vasculares retinianos. Existen también fenómenos neurodegenerativos que contribuyen a las disfunciones visuales y electrofisiológicas en estos pacientes diabéticos.

A nivel *bioquímico,* la hiperglucemia desencadena la activación de varias vías metabólicas, tales como la vía de los polioles y de la hexosamina, la síntesis de novo de proteína-quinasa C diacilglicerol (DAG-PKC), y la producción de radicales libres y productos finales de glucosilación avanzada (AGE), todas ellas importantes para el desarrollo de RD[24]. Además, existe una evidencia creciente de que los mecanismos inflamatorios también tienen un papel importante en su desarrollo[25]. La activación de todas estas vías induce un aumento en el estrés oxidativo, alteración en el flujo y en la permeabilidad vascular y la liberación de factores pro-inflamatorios y angiogénicos, tales como el factor de crecimiento vascular endotelial (VEGF). El VEGF es un mediador crucial en las complicaciones microvasculares de la DM. Es producido de forma normal por numerosas células de la retina tales como pericitos, células del epitelio pigmentario de la retina (EPR) y células de Mueller. En situaciones de hipoxia retiniana dicha producción se incrementa, existiendo una alta correlación entre el grado de RD y las concentraciones intraoculares de VEGF. Además, el VEGF tiene un papel fundamental en la patogénesis del EMD a través del aumento de la permeabilidad vascular. Como consecuencia de la activación de estas vías metabólicas, aparecen lesiones microangiopáticas en el lecho capilar, y procesos neurodegenerativos caracterizados por apoptosis neural y gliosis reactiva. Existen estudios que sugieren que esta neurodegeneración retiniana es el proceso más precoz en la patogénesis de la RD, y que participa en el desarrollo posterior de las alteraciones microvasculares[26] (Figura 1).

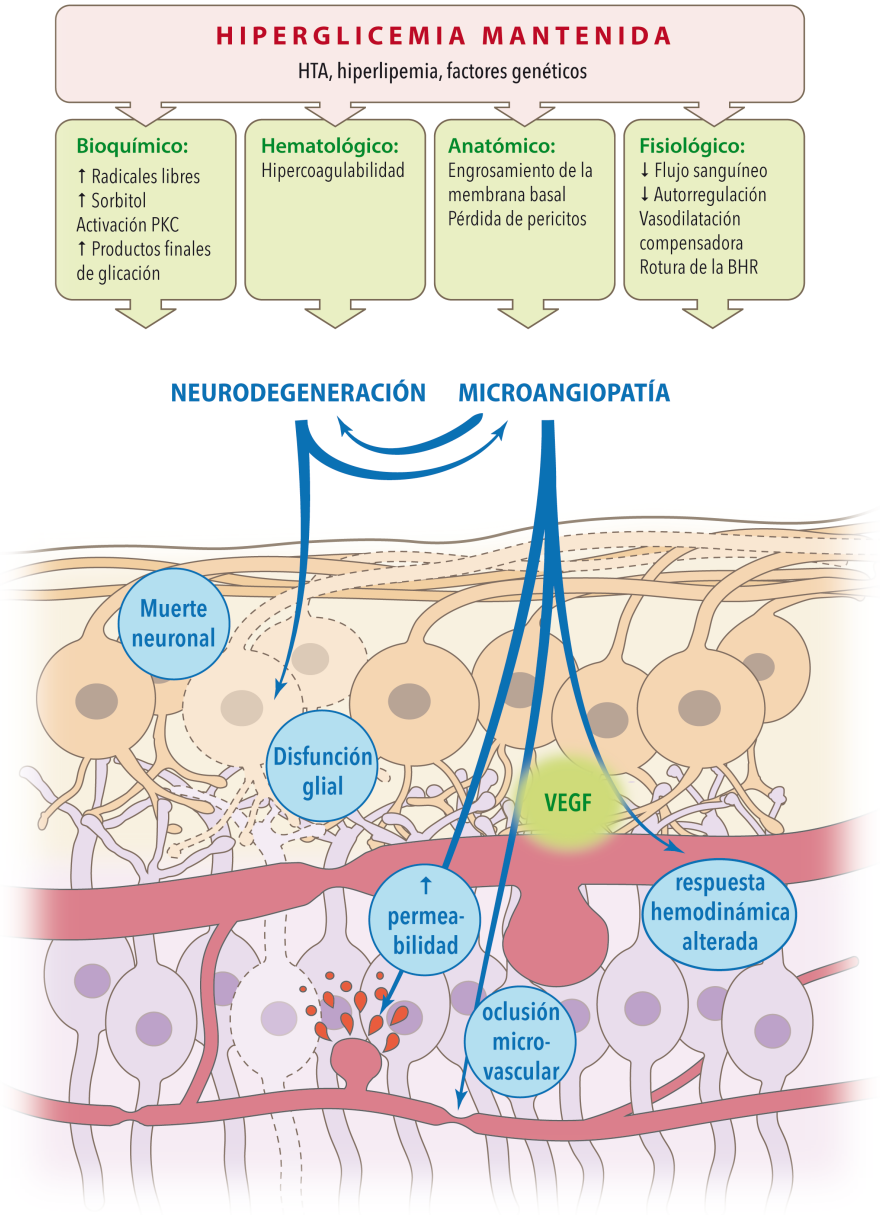

Figura 1. Representación esquemática de los principales mecanismos que conducen a la retinopatía diabética. Los cambios precoces en la retina neural incluyen la apoptosis neuronal y la disfunción glial, mientras que el aumento de la permeabilidad vascular, la regresión vascular y la alteración en la respuesta hemodinámica son las principales características de las anomalías microvasculares precoces. PKC: proteína quinasa C. VEGF: vascular factor de crecimiento vascular endotelial. HTA: hipertensión arterial. BHR: Barrera hematorretiniana.

A nivel *anatómico* aparece un engrosamiento de la membrana basal capilar que condiciona una disminución en el calibre y flujo vascular, y una proliferación de células endoteliales, con la consiguiente hipoxia tisular acompañante. Aparece también una pérdida de pericitos en los capilares de la retina, hallazgo patognomónico de la enfermedad diabética microangiopática y causante de la aparición de microaneurismas.

A nivel *hematológico* la hiperglucemia induce un estado de hipercoagubilidad, con aumento de la viscosidad sanguínea y de la adhesividad y agregación plaquetaria. Existen estudios que muestran que los glóbulos rojos de los pacientes diabéticos son más rígidos, con una menor deformabilidad y mayor agregación a nivel capilar[27-31]. Esto podría hacerlos más frágiles y susceptibles de rotura, contribuyendo así, junto con los factores anteriores, a la oclusión microvascular retinana y a la anemia que observamos en pacientes diabéticos. De hecho, bajos niveles de Hb y hematocrito se han demostrado como factores de riesgo independientes para el desarrollo y progresión de la RD[19]. Quiao et al[20] encontraron que los pacientes diabéticos con niveles de Hb inferiores a 12mg/dl, tenían dos veces más riesgo de desarrollar RD.

El estímulo isquémico es capaz de inducir, como hemos comentado anteriormente, la liberación de factores angiogénicos como VEGF. Aunque la relevancia de VEGF en la patogénesis de la RD, especialmente en las formas proliferativas, es indiscutible, se han identificado nuevas vías independientes en la patogénesis de la RD. Entre ellas encontramos la producción de eritropoyetina (EPO), factor con efecto neuroprotector inicial capaz de aumentar en estadios avanzados la proliferación de nuevos vasos retinianos y la permeabilidad vascular, contribuyendo así al desarrollo de EMD y de formas más graves de RD. Se han demostrado niveles intravítreos elevados de VEGF y de EPO en casos de RD proliferativa, pero también en pacientes con EM diabético sin isquemia y en aquellos pacientes diabéticos sin RD. Estos hallazgos sugieren que otros factores, además de la isquemia, están involucrados en su sobreexpresión en la RD[26,32-35] (Figura 2).

Así pues, la consecuencia final de todos estos cambios fisiopatológicos son lesiones microangiopáticas y neurodegenerativas responsables de los hallazgos clínicos observados en pacientes con RD. La discapacidad visual que acontece es secundaria al aumento de la permeabilidad vascular y a la proliferación de neovasos retinianos. El EM es la principal causa de pérdida visual en los pacientes con DM tipo 2[36]. La RD proliferativa puede acompañarse de hemorragias vítreas y retinianas que contribuyan a este deterioro visual.

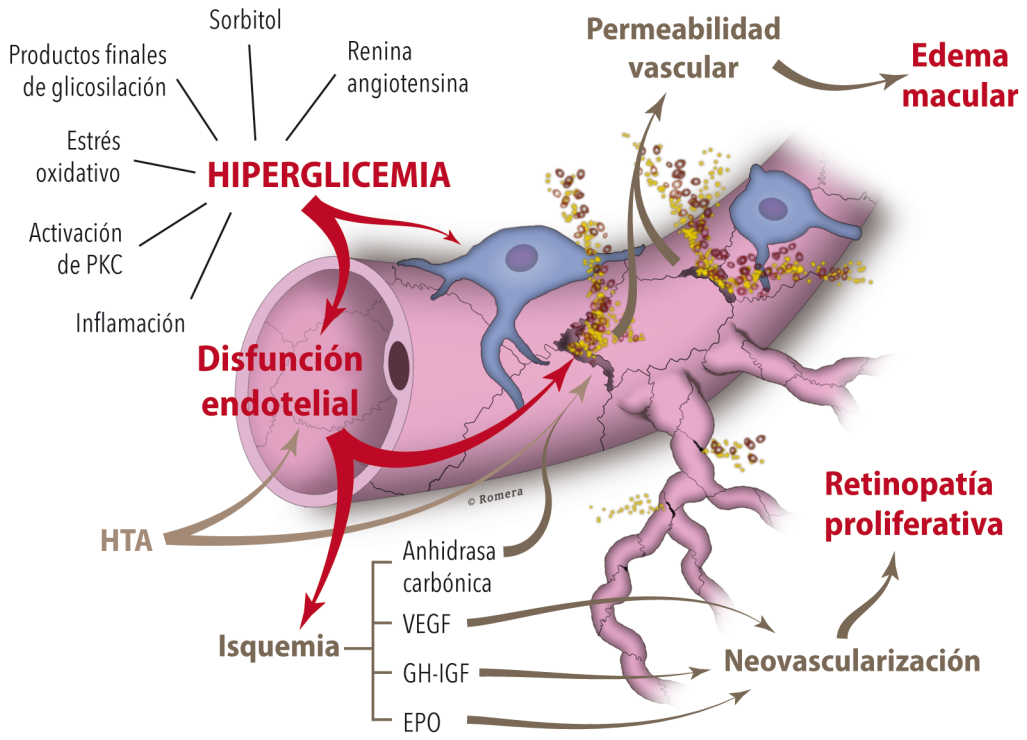

Figura 2. Fisiopatología de la retinopatía diabética. La hiperglucemia produce una cascada de eventos que conducen a la disfunción endotelial vascular retiniana. La isquemia retiniana resultante y el aumento de la permeabilidad vascular, aumentada por la hipertensión, son dos vías claves comunes en su desarrollo. PKC: proteína quinasa C. VEGF: vascular factor de crecimiento vascular endotelial. GH: hormona de crecimiento; IGF: factor de crecimiento derivado de la insulina, EPO: eritropoyetina.

2.4. La enfermedad arteriosclerótica en la diabetes tipo 2

Los pacientes diabéticos tienen un riesgo aumentado de morbilidad y mortalidad cardiovascular, sobre todo a expensas de la enfermedad coronaria. En la mayoría de los casos, los eventos cardiovasculares son producidos por el desarrollo y posterior inestabilidad de la placa de ateroma[37]. La aterotrombosis es la causa de casi el 80% de las muertes entre la población diabética, y puede manifestarse clínicamente como muerte súbita de origen cardíaco, infarto agudo de miocardio o angina, ictus isquémico o como isquemia arterial periférica[38,39].

2.4.1. Aspectos patogenéticos generales de la arteriosclerosis

La arteriosclerosis es una enfermedad arterial sistémica que afecta principalmente a la capa íntima de los vasos sanguíneos de mediano y gran calibre, incluyendo la arteria carótida, las arterias coronarias y otras arterias periféricas, como las de las extremidades inferiores[40]. El proceso aterogénico se inicia con una disfunción endotelial que favorece la entrada y oxidación de lipoproteínas de baja densidad (LDL) circulantes dentro de la pared vascular, así como la respuesta inflamatoria local, con reclutamiento de células inflamatorias. Se produce una infiltración de monocitos a nivel subendotelial, con diferenciación posterior a macrófagos y células espumosas cargadas de lípidos. Este acúmulo lipídico suele impulsar el crecimiento de la placa ateromatosa, como también lo hace la liberación de mediadores inflamatorios y de factores de crecimiento, con muerte de macrófagos y formación de un núcleo necrótico. En etapas más avanzadas aparece una proliferación de células musculares lisas y el depósito de colágeno alrededor de la placa, en forma de una capa fibrosa que estabiliza y aisla este núcleo necrótico del torrente sanguíneo. La capa fibrosa está en permanente proceso de remodelación de su contenido en colágeno, de manera que cualquier reducción en su resistencia puede aumentar la probabilidad de ruptura de la placa de ateroma. Como consecuencia del debilitamiento de la capa fibrosa, el contenido en colágeno y lípidos de la placa puede quedar expuesto al torrente sanguíneo, favoreciendo así la acumulación y la adhesión plaquetar, y la formación de coágulos de sangre que pueden bloquear el flujo sanguíneo en la arteria afectada de forma aguda, produciendo los eventos clínicos cardiovasculares[37,41-42].

La neovascularización de la placa de ateroma, que proviene de la pared vascular, es también una característica consistente en el desarrollo de la enfermedad aterosclerótica[43]. De forma fisiológica, el tercio más externo de la pared arterial, que no puede nutrirse a través de la luz arterial, recibe el aporte de nutrientes y sangre oxigenada que necesita a partir de los pequeños vasos situados en la adventicia arterial. Este plexo de microvasos en la pared arterial se llama *vasa vasorum* (VV). En los casos de arteriosclerosis, la hipoxia en la pared arterial causada bien por un engrosamiento patológico de la misma, o bien por el aumento en la

demanda de consumo de oxígeno, constituye un estímulo potente para la hiperplasia de los VV de la adventicia arterial y para la neoangiogénesis[77,41-42,44] (Figura 3).

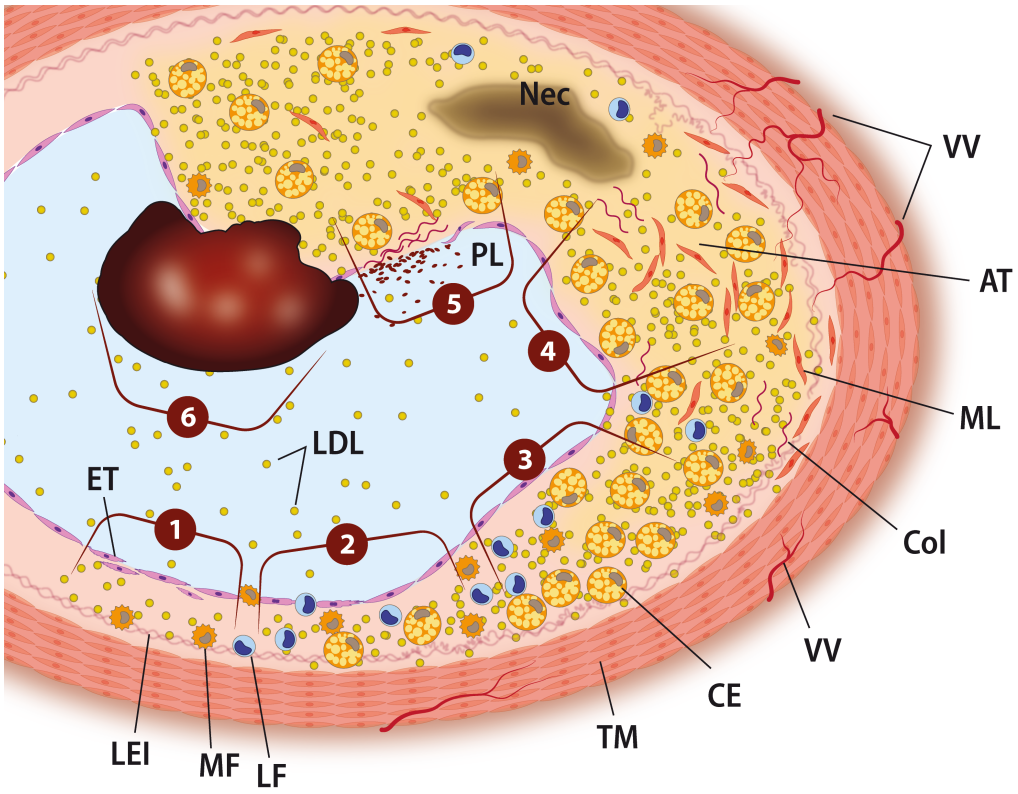

Figura 3. Proceso de ateromatosis. (1) Daño del endotelio (ET) y difusión de lipoproteínas (LDL) a la íntima arterial. (2) Migración de macrófagos (MF) y linfocitos (LF) e inicio de una respuesta inflamatoria. (3) Fagocitosis del colesterol y formación de células espumosas (CE). (4) Progresión de la placa de ateroma (AT), fragmentación de la lámina elástica interna (LEI), proliferación de células musculares lisas (ML), colágeno (Col) y neovascularización de los vasa vasorum (VV). (5) Necrosis (Nec) o hemorragia intraplaca y agregación plaquetaria (PL) en zonas de rotura de la placa. (6) Trombosis intravascular en zona de placa ulcerada. TM: túnica muscular.

2.4.2. Características de la arterotrombosis diabética

Aunque la arterioesclerosis que presentan los pacientes con DM tiene una base común a la de la población no diabética, la hiperglucemia juega un papel determinante en la aceleración y la gravedad del proceso aterogénico. Los pacientes con DM tipo 2 tienen cuantitativamente más arteriosclerosis que los pacientes no diabéticos, probablemente por la coexistencia de la diabetes con otros factores de riesgo metabólico, tales como la obesidad, la hipertensión, dislipemia o la microalbuminuria, que actúan como factores adicionales contribuyentes al desarrollo del proceso[45]. En la DM la arteriosclerosis ocurre de forma más precoz, progresa de forma más rápida y se desarrolla de forma más extensa y periférica[46].

La composición de la placa de ateroma es diferente en los pacientes con diabetes. Existen estudios que sugieren que los pacientes diabéticos que mueren por enfermedad coronaria grave presentan mayores infiltrados por macrófagos y linfocitos T en sus placas arterioscleróticas, núcleos necróticos más grandes, así como afectación ateromatosa más extensa y distal en sus arterias coronarias comparado con los pacientes no diabéticos[47]. El proceso microangiopático de arteriosclerosis está incrementado en el paciente diabético, con hallazgos estructurales y funcionales propios que incluyen la inflamación adventicia y la neovascularización aumentada de la placa. Este aumento de la neovascularización se correlaciona con la cantidad de células inflamatorias, con las hemorragias intra-placa y con la ruptura de la placa.

Todos estos factores contribuyen a una mayor progresión de la arteriosclerosis y añaden inestabilidad a la placa y, junto con el aumento en la concentración de factores procoagulantes propio de los pacientes diabéticos, aumentan la vulnerabilidad de estos pacientes a sufrir eventos cardiovasculares[37].

2.4.3. Arteriosclerosis subclínica en la diabetes tipo 2

El hecho que la población diabética tenga cuantitativamente mayor extensión de arteriosclerosis, y que ésta sea la responsable de la mayoría de muertes de los pacientes con diabetes, hace que sea fundamental el conocer y detectar los cambios precoces que acontecen en la arteriosclerosis subclínica de los pacientes diabéticos[40].

Hasta el momento se conoce que una de las lesiones más precoces en el desarrollo de la placa de ateroma es el engrosamiento del complejo íntima-media arterial (GIM) [48]. Sin embargo, y a pesar de ser un marcador extensamente validado de arteriosclerosis subclínica[48,49], el GIM puede representar un engrosamiento de la íntima, un incremento en la capa media, o bien reflejar el contenido inflamatorio que precede la aparición de la placa de ateroma[49]. A partir de estudios anatomo-patológicos post-mortem, sabemos que incluso en

estas lesiones iniciales, ya existe un incremento de VV en la adventicia de aquellos sujetos con un evento cardiovascular, respecto aquellos asintomáticos[50]. La hiperplasia de los VV es un signo precoz de arteriosclerosis, mientras que la infiltración de la pared arterial por parte de macrófagos constituye una fase más avanzada de la enfermedad.

En este sentido, la ecografía de las arterias carótidas se utiliza con frecuencia para detectar signos tempranos de aterosclerosis, tales como el aumento del grosor íntima-media de la pared arterial y la presencia de placa de ateroma, como manifestación preclínica de la enfermedad. Estudios recientes sugieren que la existencia de placa carotídea es un mejor predictor de eventos CV que las mediciones del GIM[51,52].

2.4.4. Interrelación entre la micro y la macroangiopatía diabética

A pesar de que tradicionalmente las complicaciones micro y macrovasculares de la diabetes se han visto, estudiado y tratado como trastornos distintos e independientes, una línea de pensamiento actual sugiere que existe un "terreno común" (*common soil*) para su desarrollo[53], de manera que ambos tipos de complicaciones compartirían mecanismos fisiopatológicos comunes[54-56].

Como hemos comentado anteriormente, existe una red microvascular conocida como VV que se encuentra en la adventicia de arterias de gran calibre, y que tiene como finalidad el suministro de oxígeno y nutrientes a la parte más externa de la pared vascular. Existen autores que sugieren la hiperglucemia como factor capaz de alterar la estructura y densidad de los vasa vasorum de las grandes arterias en ratones[57]. Los niveles elevados de glucemia juegan también un papel determinante en la aceleración y gravedad del proceso aterogénico. Existen estudios que demuestran una mayor inflamación adventicial y neovascularización de VV en las placas de ateroma de pacientes diabéticos[37]. Teniendo en cuenta estos hechos, y que los pacientes diabéticos con complicaciones microvasculares tienen mayor predisposición a arteriosclerosis acelerada y a muerte prematura por eventos cardiovasculares, la neovascularización derivada de los vasa vasorum podría ser el vínculo de relación de la microangiopatía con la macroangiopatía diabética. Este hecho contribuiría a explicar la precocidad, y mayor extensión y gravedad del proceso arteriosclerótico en el pacientes diabético.

Cabe destacar que los estímulos principales que conducen a la angiogénesis de los VV son la hipoxia y la isquemia de la pared vascular[58], situaciones análogas a las observadas en la retina de pacientes con RD[59]. Apoyando este hecho, existen datos obtenidos a partir de estudios epidemiológicos que demuestran que la RD, considerada como una complicación microvascular crónica común de la diabetes, se asocia con una mayor morbilidad y mortalidad CV en pacientes con DM tipo 2[60]. En estos pacientes, la presencia de RD se ha descrito como

un factor de riesgo independiente para la enfermedad coronaria incidente[61,62] y el accidente cerebrovascular isquémico[63] (Figura 4).

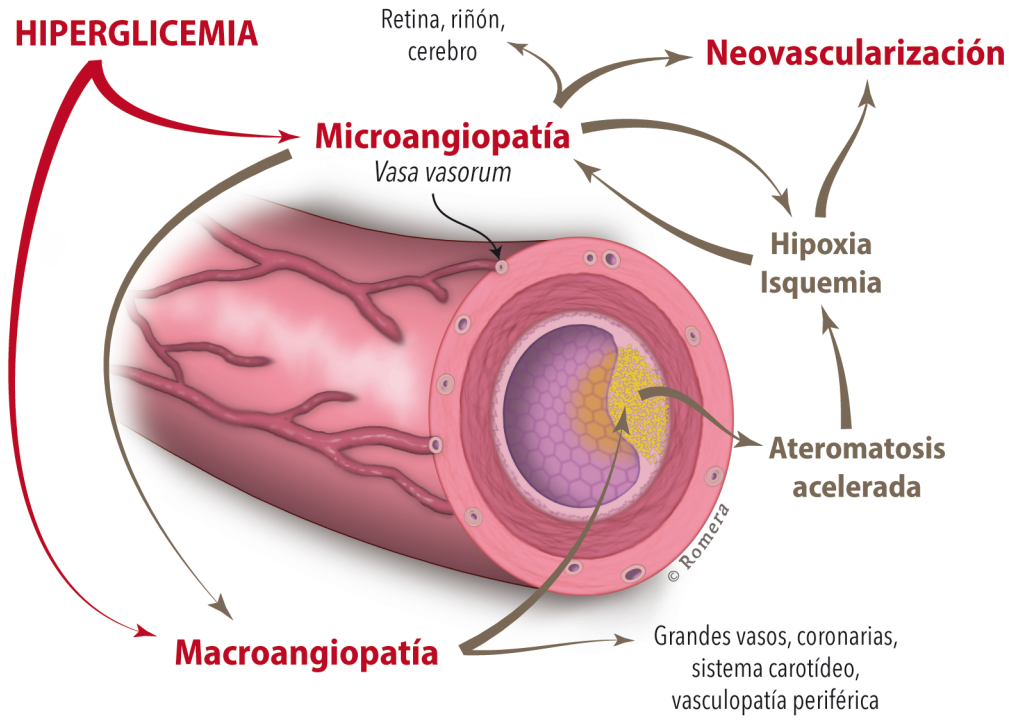

Figura 4. Relación entre la micro y la macroangiopatía en la arteriosclerosis acelerada. En la diabetes mellitus tipo 2, la angiogénesis y la microangiopatía se encuentran incrementadas, pudiendo contribuir a la arteriosclerosis acelerada que observamos en estos pacientes. La hiperglucemia puede producir afectación tanto macro como microvascular[55].

3. HIPÓTESIS DE TRABAJO

HIPÓTESIS 1

La menor capacidad transportadora de oxígeno de la sangre puede ser un factor contribuyente para el desarrollo y/o la progresión de la retinopatía diabética, la isquemia retiniana y el edema macular en pacientes con DM tipo 2.

HIPÓTESIS 2

Los cambios neurodegenerativos y del grosor retiniano pueden observarse en etapas iniciales de la retinopatía diabética, y la menor capacidad transportadora de oxígeno de la sangre puede contribuir a su desarrollo.

HIPÓTESIS 3

Planteamos que la microangiopatía diabética puede afectar a órganos no considerados clásicamente como dianas de esta complicación de la enfermedad. En concreto, planteamos la existencia de microangiopatía que afecta a los vasa vasorum de la pared de la arteria carótida de pacientes con DM tipo 2. Pensamos que existe una contribución de la enfermedad microvascular carotídea en el proceso arteriosclerótico, y que existe una asociación de esta microangiopatía de la pared arterial con la presencia de retinopatía diabética.

HIPÓTESIS 4

La presencia de retinopatía diabética se asocia con la presencia más frecuentemente de enfermedad ateromatosa subclínica carotídea en comparación con los pacientes sin retinopatía diabética, independientemente de otros factores que pueden contribuir a la arteriosclerosis.

4. OBJETIVOS

OBJETIVO 1

Evaluar si la baja capacidad de transporte de oxígeno en la sangre se asocia con la presencia y/o grado de retinopatía diabética, mediante la determinación de indicadores hematológicos (hemoglobina, hematocrito y número de eritrocitos), y su asociación con la presencia y gravedad de la retinopatía diabética, la isquemia retiniana y el edema macular en pacientes con diabetes tipo 2 sin otras complicaciones avanzadas de la diabetes.

OBJETIVO 2

Determinar el grosor macular y de la capa de fibras nerviosas de la retina en pacientes con diabetes tipo 2 sin RD y en aquellos con RD leve sin edema macular acompañante, y valorar su asociación con las concentraciones de hemoglobina, el hematocrito y el número de eritrocitos.

OBJETIVO 3

Evaluar la señal de vasa vasorum mediante ecografía con contraste de microburbujas en un segmento de la pared arterial de la carótida común libre de placa en pacientes con DM tipo 2 con y sin RD. Además, también se propuso evaluar dicha señal en comparación con la de sujetos sin diabetes.

OBJETIVO 4

Determinar la frecuencia de placa de ateroma y el grosor íntima-media en pacientes con retinopatía diabética y su comparación con pacientes sin esta complicación, todos ellos sin enfermedad cardiovascular clínica previa u otras complicaciones avanzadas de la diabetes.

5. ARTÍCULOS

Para poder responder a las hipótesis y objetivos anteriores, diseñamos un estudio exploratorio, prospectivo, observacional, de casos y controles, compuesto por una población de sujetos con DM tipo 2 con retinopatía (casos) y sin retinopatía diabética, ni otra manifestación de afectación microvascular diabética (controles). Se seleccionaron pacientes de edades entre 40 y 75 años, agrupados en intervalos de 5 años, para lograr una distribución uniforme de la edad. Definimos como pacientes con evidencia de microangiopatía aquellos sujetos con retinopatía diabética de cualquier grado, con o sin neuropatía o microalbuminuria, pero sin nefropatía diabética establecida, excluyéndose los sujetos con macroalbuminuria (albúmina: creatinina orina > 299 mg / g) o insuficiencia renal (filtrado glomerular calculado < 60 ml/min). El grupo sin microangiopatía incluyó los sujetos sin evidencia de retinopatía y con normofunción renal y excreción de albumina urinaria dentro de la normalidad (< 30 mg/g creatinina). Esta estrategia permitió descartar aquellos sujetos con DM tipo 2 y con microalbuminuria, que en una proporción muy significativa no progresan a nefropatía y, por tanto, no pueden ser considerados como portadores de microangiopatía diabética en sentido estricto. Con esta aproximación se garantizó al máximo la definición de los grupos a estudiar. Se incluyeron pacientes con DM tipo 2 sin evidencia de enfermedad vascular cerebral previa, ni evidencia de enfermedad coronaria previa (que incluye las clases III/IV de insuficiencia cardiaca de la New York Heart Association (NYHA)), ni enfermedad arterial periférica en extremidades inferiores (incluyendo historia previa de pie diabético). El reclutamiento primario de los sujetos se realizó en el Servicio de Oftalmología. Los pacientes fueron identificados a partir de visitas realizadas en la unidad de retina o a través del sistema de cribado virtual de RD.

La clasificación de la RD se realizó siguiendo la Clasificación clínica Internacional de la RD y el Edema Macular[4]. En la anamnesis, se recogieron datos clínicos sobre antecedentes familiares de DM, enfermedad cardiovascular precoz, dislipemia e hipertensión arterial. En relación a la DM, se identificó la fecha, edad de diagnóstico y los criterios de clasificación. Se recogió también historia de tabaquismo, medicación activa, dislipemia, hipertensión arterial, y datos demográficos. Entre las variables de exploración física, se incluyó una exploración física completa, con determinación del peso, talla, perímetro de cintura, y presión arterial. Se valoraron también las variables analíticas rutinarias: glucemia, hemoglobina glucosilada, estudio de lípidos y sus fracciones, función renal, excreción urinaria de albúmina, hemograma completo y proteína C reactiva, entre otros.

5.1. ARTÍCULO 1

Para valorar si la baja capacidad de transporte de oxígeno en la sangre puede ser un factor contribuyente a los cambios observados en la RD, incluimos 312 pacientes diabéticos tipo 2 según criterios anteriores, y los clasificamos en función de la presencia o ausencia de RD y de su grado. Desde un punto de vista oftalmológico, se excluyeron aquellos pacientes con errores refractivos o patología ocular concomitante que pudieran dificultar el análisis de datos. Se realizó una exploración oftalmológica completa en todos los pacientes, que incluyó la toma de la mejor agudeza visual corregida, determinación de la presión intraocular, y exploración del fondo de ojo bajo dilatación pupilar. Se realizaron retinografías funduscópicas y angiografía fluoresceínica en todos aquellos pacientes con algún grado de retinopatía diabética. Se valoró la presencia o ausencia de edema macular clínicamente significativo y de isquemia retiniana, definida por la presencia de isquemia macular y/o periférica acompañante. Correlacionamos estos hallazgos oftalmológicos con las variables generales y hematológicas analizadas.

LOWER HEMOGLOBIN CONCENTRATION IS ASSOCIATED WITH RETINAL ISCHEMIA AND THE SEVERITY OF DIABETIC RETINOPATHY IN TYPE 2 DIABETES

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ABSTRACT

- PURPOSE: To assess the association between hematological variables with the prevalence of diabetic retinopathy, retinal ischemia and macular oedema in patients with type 2 diabetes mellitus.
- DESIGN: Cross-sectional, case-control study
- METHODS: Case-control study with 312 individuals with type 2 diabetes mellitus: 153 individuals with diabetic retinopathy (cases) and 159 individuals with no diabetic retinopathy (controls). Participants were classified according to the severity of diabetic retinopathy and the presence of retinal ischemia or macular oedema. In addition, hematological variables (i.e. haemoglobin, haematocrit and erythrocytes) and cardiovascular risk factors were collected by standardized methods. Three logistic models were adjusted to ascertain the association between hematologic variables with the severity of retinopathy and the presence of retinal ischemia or macular oedema.
- RESULTS: The bivariated analysis showed significantly lower haemoglobin, hematocrit and erythrocyte levels were observed in individuals with severe diabetic retinopathy compared with those with mild disease, and in individuals with retinal ischemia and macular oedema compared with those without these disorders. However, haemoglobin was the only factor that showed a significant inverse association with the severity of diabetic retinopathy [beta coefficient=-0.52, p-value=0.003] and the presence of retinal ischemia [beta coefficient=-0.49, p-value=0.001]. Lower erythrocyte level showed a marginally significant association with the presence of macular oedema [beta coefficient=-0.86, p-value=0.055].
- CONCLUSIONS: Low blood oxygen-transport capacity, determined by decreased heamoglobin levels, was associated with more severe diabetic retinopathy and the presence of retinal ischemia. These results suggested that low haemoglobin levels may have a key role in the development and progression of diabetic retinopathy.

Key Words: hemoglobin, type 2 diabetes, diabetic retinopathy, retinal ischemia

INTRODUCTION

Type 2 diabetes mellitus (DM) is a common disease with a prevalence and incidence that have increased in recent decades. The estimated increase in the total number of people with diabetes is from 366 million in 2011 to 552 million in 2030 [1]. Diabetic retinopathy (DR), including diabetic maculopathy, is a microvascular complication of DM and the leading cause of visual disability in adults of working age in developed countries. Its prevalence increases with age and the duration of diabetes. It is estimated that after 20 years of having diabetes, more than 20% of type 2 diabetic patients will have some degree of DR[2].

The initial changes in DR affect the microcirculation of the retina. The lesions observed in these patients are secondary to this microangiopathy and result in increased vascular permeability in the form of edema and retinal vascular occlusive disease. The retina is among the most active metabolic tissues of the human body and is highly sensitive to reductions in oxygen tension [3]. Some studies have suggested that hypoxia is a stimulus for the production of intraocular and systemic erythropoietin (EPO) [3-5] and for vascular endothelial growth factor (VEGF). Both EPO and VEGF are neuroprotective factors that possess likely angiogenic potential, leading to an increase in the proliferation of new retinal vessels and thus inducing the development of proliferative retinopathy.

Macular edema (ME) may appear at any stage of DR and is the main cause of central visual loss in diabetic patients. It is defined as an increase in tissue fluid, which causes a thickening of the retina and secondarily causes structural and functional alterations with important clinical consequences. According to data from the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) [6], after 15 years of known diabetes, the prevalence of diabetic macular edema is approximately 25% and 14% in patients with type 2 DM treated with and without insulin, respectively.

Despite not fully understanding the pathogenic mechanisms of DR and ME, there are a large number of multicenter studies that have assessed the risk factors involved in DR and ME development and progression [7-11]. The duration of diabetes, glycemic control and the systolic blood pressure are the most accepted and widely documented risk factors.

Several authors have also suggested that hemoglobin, hematocrit and blood viscosity levels can contribute to the development and progression of DR[12-16]. Low hemoglobin was an independent baseline risk factor for the development of proliferative diabetic retinopathy (PDR) and severe visual loss in the Early Treatment Diabetic Retinopathy Study Report (ETDRS)[12]. Other studies have corroborated this finding and found an improvement in DR status following a correction of the anemia. [13.17 -19] A prospective study published by *Qing Qiao et al* in 1997 showed an inverse independent relationship between hemoglobin concentrations and the development of DR, particularly severe forms of DR [13].

Similarly, we sought to assess whether a lower blood-oxygen transport capacity may be associated with the presence and severity of retinal disease in type 2 diabetes. To accomplish this, we determined the potential association of hematological factors, particularly the hemoglobin concentration, with the presence and severity of diabetic retinopathy, retinal ischemia and macular edema in patients with type 2 diabetes.

METHODS

• STUDY POPULATION: This was a prospective, observational, exploratory study of cases (n=153) and controls (n=159). It included subjects with type 2 DM with retinopathy (cases) and without DR (controls). This study was initially designed to evaluate the presence of subclinical micro and macrovascular disease associated with diabetic retinopathy. In a recent publication, we described the increased frequency of subclinical atherosclerosis associated with diabetic retinopathy [20]. We took advantage of this study in which 312 type 2 diabetic patients aged 40-75 years without known clinical cardiovascular disease or renal failure (glomerular filtration rate < 60 ml/min) were included. The presence or absence of diabetic retinopathy was used for the allocation of the study groups by study design. To avoid including patients with additional microangiopathic burden, patients without retinopathy also had a current and previous normal urinary albumin: creatinine ratio (<30 mg/g). In the subgroup of diabetic patients with retinopathy, macroalbuminuria (urine albumin: creatinine ratio > 299 mg/g) was an additional exclusion criterion. We selected patients of several different ages to achieve a similar age distributions in the groups.

All of the diabetic patients were recruited from the outpatient clinic at our center. Potential candidates were identified from the screening and treatment diabetic eye disease program of our center. The clinical characterization of these patients has been recently described [20]. From an ophthalmological point of view, we also considered the following as exclusion criteria: refractive errors (spheric equivalent)> + /- 3.00 diopters; average opacity that made data analysis difficult; presence of other concomitant retinal pathology; surgery during the previous year; inflammatory conditions in the anterior or posterior segment; glaucoma treatment; and laser photocoagulation within the previous 6 months. The local Ethics Committee approved the study, according to the Helsinki Declaration. All patients signed an informed consent form.

• DEFINITION OF VARIABLES: All patients underwent a clinical evaluation that included: age, sex, ethnicity, antidiabetic treatment, treatment for dyslipidemia and hypertension, use of antiplatelet drugs, systolic and diastolic blood pressure, body mass index, emoglobin HbA1c, lipid profile, creatinine and urinary albumin/creatinine ratio. Data on these variables from the

study subjects has been already published [20], and are used here for the purpose the analysis of the present study outcomes The methodology used to assess the clinical variables is described in detail elsewhere [20]. For the specific purpose of the current study, a blood count that included hemoglobin, hematocrit, and erythrocytes was conducted with the model TOASYSMEX XN-20 device (Roche, Japan).

• OPHTHALMIC EXAMINATION: Patients underwent a complete eye examination to assess the presence or absence of diabetic retinopathy, clinically significant macular edema and retinal ischemia.

According to a modified version of the American Academy Ophthalmology classification [21], the patients were classified according to the presence or absence of diabetic retinopathy (DR) and its severity: absence of DR (NDR), mild non-proliferative DR (NPDR), moderate to severe NPDR and proliferative diabetic retinopathy (PDR). For the statistical analyses, we categorized the presence of diabetic retinopathy into 2 groups: group 1, mild DR and group 2, DR> mild (including moderate DR, severe, or PDR).

Clinically significant macular edema (CSME) was defined according to the Early Treatment Diabetic Retinopathy Study (ETDRS) classification protocol: the presence of retinal thickening at or within 500 μm of the center of the macula, hard exudates at or within 500 μm of the center of the macula associated with thickening of the adjacent retina, and/or zones of retinal thickening 1 disc area in size and at least partially within a 1-disc diameter of the center[22].

The ophthalmologic examination included an automatic refraction (MRK-3100P, Huvitz), collection of the best corrected visual acuity, measurement of intraocular pressure (applanation tonometer Measured By model AT 900, Haag-Streit), exploration with slit-lamp of the anterior segment and pupil dilation with 1% tropicamide (Superfield NC and 90D and +78D Volk lens).

Visual acuity in each eye was measured on the Snellen chart and recorded as a decimal value with the best refraction for distance, and the data were applied to the logMAR (logarithm of the minimum angle of resolution) ETDRS chart. All data handling on visual acuity is expressed in logMAR format.

The fundi of all the patients were photographed using a 45° field stereoscopic digital fundus camera (TOPCON TRC: 50IX, retinal camera), centered first from the temporal to the macula and second from the nasal to the papilla. [23] For those who showed evidence of any retinopathy, additional 30° seven-field stereo digital pairs were taken.

The patients with identified diabetic retinopathy underwent a fluorescein angiography. The fluorescein angiography was performed according to a standard procedure and was used to assess the foveal avascular zone (FAZ) and peripheral retinal ischemia according to the ETDRS standards[24]. Two 30° fields extending along the horizontal meridian from 25° nasal to

the disc to 20° temporal to the macula and four 45° peripheric fields were selected for further analysis. The presence of retinal ischemia was assessed by the capillary loss both in the peripheral areas and in the central subfield. The patients were classified into two groups: group 1, which had an absence of retinal ischemia, and group 2, in whom retinal ischemia was present (macular or peripheral to any degree).

An optical coherence tomography (OCT) was carried out in all the patients who permitted to evaluation of the macular area. The OCT used was the *Stratus Optical Tomography 3 (Carl Zeiss Meditec, Dublin, CA, USA).* The analysis of results was carried out using the *Fast macular Thickness Map.*

• STATISTICAL REVIEW: The continuous variables are presented as the mean and interquartile range, and the categorical variables are presented as proportions. We used the Mann-Whitney U and Chi-square tests to compare the means and the proportions of the risk factors for diabetic retinopathy and hematological factors (hemoglobin (Hb), hematocrit (Htc), erythrocytes, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), according to the presence of DR, the presence of mild DR vs. DR>mild (moderate/severe/proliferative), the presence of retinal ischemia vs. absence of retinal ischemia and the presence of EMCS vs absence of EMCS.

Three logistic regression models for each of the variables considered were used (mild DR vs. DR>mild, retinal ischemia and EMCS). The variables that showed a statistically significant association in the bivariate analysis with each of the variables of interest were used as potential confounders. The adjustment of the logistic regression models followed the stepwise procedure, and those that showed a significant association with the variables of interest were adjusted. The saturated models mild DR vs DR>mild and retinal ischemia were adjusted by hemoglobin, sex and the interaction sex-hemoglobin. Finally, the EMCS saturated model was adjusted by erythrocytes, sex and sex interaction-erythrocytes. We tested whether there was an interaction between hemoglobin and sex for each of the variables of interest (mild DR vs. DR>mild, the retinal ischemia and the EMCS). A univariate logistic regression model was adjusted, in which the association was estimated and stratified by sex of the DR patient with hemoglobin for the variables that exhibited a significant interaction.

RESULTS

• CHARACTERISTICS OF STUDIED SUBJECTS: As already explained above, the general clinical characteristics of the study groups has already been published [20]. The assessment of the characteristics of the study groups showed that, compared with the subjects

without retinopathy, the diabetic subjects with retinopathy had a longer diabetes duration, higher HbA1c concentration, and higher prevalence of hypertension, systolic blood pressure and urinary albumin excretion. The patients with retinopathy had also greater waist circumference and HDL-c. Additionally, they were more likely to be on insulin treatment and on antiplatelet drugs.

There were no differences in any of the hematological variables between patients with and those without retinopathy (Table 1). Additionally, no significant differences were observed between the concentrations of iron and ferritin (Table 1).

The distribution of retinopathy grades were as follows: 60 patients had mild NPDR (39.2%), and 93 patients NPDR > mild (57 moderate NPDR (37.3%), 29 severe NPDR (18.9%), and 7 PDR (4.6%)). The patients with retinopathy were slightly older. The proportion of male/female subjects was not different between the groups.

• RELATIONSHIP AMONG VARIABLES OF INTEREST, GENERAL FACTORS AND HEMATOLOGICAL VALUES: The patients with a degree of diabetic retinopathy greater than mild (moderate/severe/proliferative) presented with higher glycated hemoglobin, systolic blood pressure, triglycerides, urinary albumin excretion and were more likely on insulin treatment. Hb, Htc and erythrocytes were significantly decreased with the increasing severity of DR. Table 2 shows the variables that were statistically significant in the comparison between the groups.

Among the 153 study patients with DR, 58 had some degree of retinal ischemia (macular and/or peripheral). These patients were older, had a longer diabetes duration, were more likely to be on insulin treatment and had a higher prevalence of microalbuminuria. The patients in the group without retinal ischemia were more often smokers, but this was not statistically significant (p= 0.0621). Hb, Htc and erythrocytes were significantly reduced in the group with retinal ischemia compared with the group without retinal ischemia. (Table 3)

In patients with clinically significant macular edema (n=54), higher systolic blood pressure and urinary albumin excretion was observed. The levels of Hb, Htc and erythrocytes were significantly reduced in this group compared with the patients without macular edema (Table 4).

• RELATION BETWEEN DR, RETINAL ISCHEMIA, CSME AND HEMATOLOGICAL VARIABLES: The results of the multivariate models showed that lower levels of hemoglobin were associated with the severity of DR and the presence of retinal ischemia. In the case of macular edema, the association with the lower number of erythrocytes was significant. In addition, a significant interaction between the level of hemoglobin and sex, depending on the degree of diabetic retinopathy, was observed (Table 5). The multivariate analysis stratified by gender for the model of mild DRNP vs > mild DRNP yielded a significant hemoglobin and sex

interaction, with higher levels of hemoglobin being against diabetic retinopathy in women [Odds Ratio (confidence interval 95 %) =0.374 (0.205 -0.682); p: 0.001], whereas in men, the odds ratio of this association was not significant.

DISCUSSION

The results of our study failed to demonstrate an independent association between the concentration of Hb and the presence of DR. However, we found that lower Hb is associated with severe forms of DR and with the presence, until now not described, of retinal ischemia. We also demonstrated for the first time an association between low levels of red blood cells and the presence of EMCS.

Different factors have been described to be associated with the development and severity of DR in patients with type 2 DM [7-11]. These include the duration of DM, insulin treatment, high blood pressure, higher serum lipids, and high glycated hemoglobin urinary albumin excretion. Our study coincides with those published earlier and yields the results of a sample of patients without other advanced diabetic complications.

Anemia is suggested as another long-term complication of DM and, together with ischemia, has been associated with the development and progression of both microvascular and macrovascular complications. Ischemia is a critical component of DR, and it is potentially influenced by anemia, the main indicator of the blood oxygen delivery capacity[25]. Previous studies have suggested that compared with normal red blood cells, the erythrocytes of diabetic patients have decreased deformability and increased aggregation at the capillary level[26-30]. This condition could make them more fragile and susceptible to breakage, which would lower the hemoglobin levels. This finding may suggest that hypoxia that occurs as a result of anemia acts as a stimulus for the release of inflammatory mediators and vasoproliferative factors, such as VEGF and EPO, that are capable of increasing vascular permeability and contribute to the development of ME and more severe forms of DR. High intravitreal levels of EPO have been shown in cases of proliferative DR and in patients with diabetic ME [3-5.]

There are several studies showing the role of anemia as an independent risk factor for diabetic retinopathy, particularly in population-based studies [12-13, 15,26]. Among them, David et al [12] demonstrated that in ETDRS, low hemoglobin and hematocrit levels were independent baseline risk factors for the development of high-risk proliferative diabetic retinopathy and severe vision loss over a 5-year follow-up. *Qing Qiao et al* [3] found that diabetic patients with hemoglobin levels below 12 mg/dl were two times more likely to develop DR. A similar risk of anemia with severe retinopathy was also reported in a case series by Shorb [26]. Irace et al.[15] showed that the levels of blood viscosity, Htc and hemoglobin were lower in patients with DR

compared with subjects without retinopathy[15]. In addition, the levels of these hematological variables decreased as the degree of retinopathy increased. In that study, 190 type 2 diabetic patients both with and without DR were compared with 95 controls without DM. Premenopausal women and patients with renal failure were excluded. However, neither retinal ischemia nor macular edema were considered.

Other studies in the literature consider anemia to be a predictor of DR [11,14,17]. Ranil et al. [17] showed that type 2 diabetic patients with anemia were 1.80 times more likely to develop diabetic retinopathy than were individuals without anemia. In contrast to our study, this article did not analyze a control group without retinopathy, nor was renal function accounted for. *Karoli et al.* [11] did consider a study population of type 2 diabetes with normoalbuminuria and normal glomerular filtration and established that low Hb levels are a risk factor for the development of DR. We did not find an association between low levels of Hb and the presence or absence of DR. This is most likely explained by the design of our study and the number of patients in the control group.

Anemia and low hematocrit have been found to also be associated with the presence of diabetic macular edema. [31-32] In our study, we have corroborated these results, and we demonstrated an independent association between low levels of erythrocytes and the presence of EMCS.

There were several limitations in the present study that should be noted here because they may affect the generalization of our findings. Because this was a cross-sectional study, the results do not provide information on the potential causation effect of hemoglobin. Prospective and larger studies are required to overcome this limitation. Further, the results of this study should be interpreted with caution because it was not designed primarily to respond to these associations. The evaluation of the association between hematological parameters and the presence and severity of DR was a secondary objective. For this reason, though the majority of the patients included in the study were postmenopausal, this variable was not evaluated.

One of the strengths of this study is its case-control design, which enabled us to assess whether there are differences between patients with and without DR. The specific exclusion of other complications associated with diabetes, such as nephropathy and cardiovascular disease, provides us with a study population that is free of microvascular and microvascular disease, with the exception of the retinopathy. This eliminates the influence of these factors, especially renal insufficiency, as confounding variables and differentiates this study from the majority of the previously published studies.

In conclusion, there is an association of low blood oxygen-transport capacity, measured by hemoglobin concentration, and an increased severity of DR and the presence of retinal ischemia. These findings might have clinical implications, such as in attempts to better control

hemoglobin concentrations in diabetic patients, particularly in those with advanced forms or DR ischemia. In patients with these conditions, it would be advisable to monitor and prevent anemia. Prospective studies specifically designed to test these associations are needed to confirm low values of hemoglobin as a risk factor for DR and retinal ischemia also in patients with type 1 DM. Clinical trials in patients with anemia are needed for the further confirmation of these associations.

ALL AUTHORS HAVE COMPLETED AND SUBMITTED THE ICMJE DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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Contributions to Authors: AT participated in the study design, and wrote and drafted the manuscript. VB, CJ, RE and JE performed the ophthalmological examination. All of the authors, except for DM and EO, contributed to the data collection. EO performed the statistical analysis and reviewed the manuscript. DM conceived the study, participated in its design and coordination and reviewed the manuscript. All of the authors contributed to the discussion and approved the final manuscript. Other Acknowledgments: Maria Grau MD, PhD, Cardiovascular Epidemiology and Genetics, IMIM – Hospital del Mar Medical Research Institute, Barcelona (Spain).

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TABLE 1. Hematological variables of the study groups according to presence or absence of diabetic retinopathy (DR)

	Without DR (n = 159)	With DR (n = 153)	*P* Value
Leukocytes (x10x9/L)	6670 (5600-7730)	6795 (5685-8035)	0.3765
Hemoglobin (gr/dl)	13.8 (13-14.6)	13.6 (12.8-14.8)	0.5435
Erythrocytes (x10x12/L)	4.6 [4.4-5.0]	4.6 [4.4-4.9]	0.9407
Hematocrit (%)	41.10 [39-43.9]	40.95 [38.8-43.25]	0.7114
MCV (fl)	87.8 [85.1-90.5]	87.6 [85.0-90.1]	0.6169
MCH (pg)	29.4 [28.4-30.6]	29.3 [28.0-30.7]	0.4814
MCHC (g/dl)	33.5 [32.8-34.1]	33.4 [32.7-34.3]	0.7543
Fe (μg/dl)	78 (63-99)	74 (58-96)	0.2534
Ferritin (ng/ml)	131.2 (55.5-227.8)	98.5 (38-211.9)	0.0624

Data are presented as medians and interquartile ranges. BP: blood pressure. MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration

TABLE 2. General and hematological variables that showed statistically significant differences in the comparison between groups according to severity of diabetic retinopathy

	Mild diabetic retinopathy (n = 60)	> mild diabetic retinopathy (n = 93)	*P* Value
Serum glucose (mg/dL)	150 [113-181]	175 [135-217]	0.012
Systolic BP (mm Hg)	139 (125-147)	149 (135-164)	0.0008
Insulin treatment (n)	2 (13.88%)	16 (10.45%)	0.0020
Triglycerides (mmol/l)	138 [88-177]	110 [81-153]	0.041
Urinary albumin: creatinine ratio, mg/g	8.21 [4.84-18.8]	18.7 [7.3-39.6]	0.003
Leukocytes (x10x9/L)	7.40 [6.11-8.39]	6.42 [5.54-7.84]	0.050
Hemoglobin (gr/dl)	14.0 [13.2-15.0]	13.4 [12.6-14.4]	0.005
Erythrocytes (x10x12/L)	4.8 [4.6-5.0]	4.6 [4.4-4.9]	0.007
Hematocrit (%)	42.3 [40.0-43.8]	40.4 [38.4-42.1]	0.005

TABLE 3. General and hematological variables that showed statistically significant differences in the comparison between groups according to the presence or absence of retinal ischemia

	Absent retinal ischemia (n = 95)	Present retinal ischemia (n = 58)	*P* Value
Urinary albumin: creatinine ratio, mg/g	9.4 [5.2-21.4]	25.0 [8.4-76.2]	0.002
Disease duration (yr)	10 (5-15)	16.5 (8-23)	0.0015
Insulin treatment (n)	40(42.1%)	43 (74%)	0.038
Smoking (current/past/never)	7 (12.1%)	23 (24.5%)	0.0621
Hemoglobin (gr/dl)	14.0 [13.1-14.9]	13.1 [12.5-13.9]	<0.001
Erythrocytes (x10x12/L)	4.8 [4.5-5.0]	4.6 [4.3-4.9]	0.002
Hematocrit (%)	41.7 [39.9-43.8]	39.7 [36.6-41.4]	<0.001

TABLE 4. General and hematological variables that showed statistically significant differences in the comparison between groups according to the presence or absence of CSME

	Diabetic retinopathy without CSME (n = 159)	Diabetic retinopathy with CSME (n = 54)	*P* Value
Urinary albumin: creatinine ratio, mg/g	9.2 [5.1-22.0]	23.3 [9.4-68.8]	<0.001
Systolic BP (mm Hg)	141 (129-154)	148 (135-165)	0.0337
Hemoglobin (gr/dl)	13.8 [13.0-14.9]	13.2 [12.6-14.1]	0.047
Erythrocytes (x10x12/L)	4.7 [4.5-5.0]	4.6 [4.3-4.8]	0.019
Hematocrit (%)	41.3 [39.2-43.5]	40.3 [38.3-42.0]	0.047

TABLE 5. Association between hematologic variables and Mild DRNP vs > Mild DRNP, ischemia vs. no ischemia and CSME vs. no CSME

	Mild DRNP vs > Mild DRNP				Ischemia				CSME			
	Model 1		Model 2		Model 1		Model 2		Model 1		Model 2	
	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value
Hemoglobin	-0.36	0.005	-0.52	0.003	-0.42	0.004	-0.49	0.001	--	--	--	--
DM duration	--	--	--	--	0.04	0.038	--	--	--	--	--	--
Sex	--	--	6.31	0.009	--	--	1.19	0.565	--	--	-2.40	0.254
Sex*hemoglobin	--	--	-0.46	0.009	--	--	-0.09	0.554	--	--	--	--
Erythrocytes	--	--	--	--	--	--	--	--	-0.93	0.030	-0.86	0.055
Sex*Erythrocytes	--	--	--	--	--	--	--	--	--	--	0.48	0.284

Models mutually adjusted

5.2. ARTÍCULO 2

Con el objetivo de valorar el grosor macular y de la capa de fibras nerviosas de la retina en etapas precoces de la retinopatía diabética, seleccionamos aquellos pacientes sin signos de RD y aquellos con RD leve pero sin edema macular acompañante. En ellos realizamos una exploración oftalmológica completa con valoración adicional del área macular y peripapilar con OCT. Valoramos dichos resultados y los correlacionamos con los biomarcadores sanguíneos.

EARLY CHANGES IN RETINAL THICKNESS AND ITS ASSOCIATION WITH HEMATOLOGIC FACTORS IN TYPE 2 DIABETES MELLITUS

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ABSTRACT

Objectives. To assess whether diabetic individuals with mild retinopathy show changes in the thickness of the macula and retinal nerve fiber layer (RNFL), compared to those without retinopathy, and whether these changes are associated with hematologic factors (hematocrit, hemoglobin and erythrocytes).

Methods. Cross-sectional study of patients with type 2 diabetes mellitus with and without mild diabetic retinopathy (DR), and no clinically significant edema. We conducted an ophthalmologic examination to determine macular thickness and RNFL by Stratus optical coherence tomography (OCT). We tested for association between ophthalmologic variables and haematologic (hematocrit, hemoglobin and erythrocytes) and cardiovascular risk factors using logistic regression models adjusted for potential confounders.

Results. We included 216 individuals, 159 without DR and 57 with mild DR. Macular thickness and volume were significantly higher in individuals with mild DR than in those without DR (beta coefficient=0.016; p-value=0.022 and beta coefficient=19.837; p-value=0.025, respectively). While there was no significant difference in RNFL thickness between these groups, we observed a significant association between RNFL thickness and hematologic variables, particularly erythrocytes [beta coefficient=4.376; p-value=0.007] and hematocrit [beta coefficient=0.430; p-value=0.031]. Thus, low erythrocyte and hematocrit levels were associated with low RNFL thickness in all patients, independent of DR.

Conclusions. Changes in macular thickness are observed during the initial stages of diabetic retinopathy. Low blood oxygen-transport capacity, as determined by hematocrit, hemoglobin and erythrocytes, is associated with early neurodegenerative changes.

INTRODUCCION

Damage to retinal vessels is one of the most common complications of diabetes, and leads to the development of diabetic retinopathy (DR). In addition to vascular changes, the earliest stages of diabetic retinopathy are also characterized by loss of ganglion cell bodies, glial reactivity, and reduced thickness of the Retinal Nerve Fiber Layer (RNFL)[1-3].

Several studies have assessed macular alterations using optical coherence tomography (OCT) during the initial stages of DR[4-6]. Diabetic patients without DR have been found to have similar retinal thickness to that in non-diabetic subjects with a normal retina[4], whereas diabetic

patients with mild DR show increased macular thickness[5-6]. Thickening of the macula during the initial stages of DR suggests an early increase in vascular permeability preceding overt macular edema.

The observed decrease in RNFL during early DR indicates a loss of ganglion cell bodies and axonal degeneration. Several studies suggest that retinal neurodegeneration is the earliest process in the pathogenesis of DR, occurring even before the onset of microvascular changes, and may actually participate in the development of these changes[7-9]. New generations of OCT, including Stratus OCT, assess the thickness and volume not only of the macula, but also of the RNFL. While the pathogenic mechanisms that lead to DR are not fully understood, hemoglobin concentrations, hematocrit and blood viscosity have been proposed as potential contributors to the development and progression of DR[3]. However, previous studies that used OCT to assess early changes in retinal thickness did not consider hematological and cardiovascular risk factors that are generally altered in diabetic patients, such as hematologic variables or lipid profile[2,7,9.] We hypothesize that early changes in retinal thickness and alterations in blood biomarkers can be observed during the early stages of DR.

Therefore, the objectives of this study were: (1) to ascertain whether individuals with Type 2 Diabetes and mild DR show changes in macular volume and thickness and RNFL thickness compared to those without DR; and (2) to assess whether hematological and cardiovascular risk factors are associated with changes in macular and RNFL thickness in individuals with mild DR compared with those without DR.

METHODS

We performed a single-centre cross-sectional study of 216 patients with type 2 diabetes mellitus, 159 without DR and 57 with mild DR. Candidate subjects were identified from an ongoing screening and treatment program for diabetic eye disease at our center. The study protocol is described in detail elsewhere[10].

The inclusion criteria for both groups were as follows: age range, 40-75 years; absence of established impaired renal function (calculated glomerular filtration rate (eGFR) <60 ml/min), absence of macroalbuminuria (albumin/creatinine ratio >300mg/g, and absence of established cardiovascular disease. All participants underwent a complete physical examination and blood analysis to collect data on the following variables: age, sex, ethnicity, treatment for diabetes, dyslipidemia and hypertension, use of antiplatelet drugs, systolic and diastolic blood pressure, body mass index, glycated hemoglobin (HbA1c), lipid profile, creatinine and urinary albumin/creatinine ratio. Data for these variables for the study subjects has been published previously[10], and are used here to analyze the outcomes of interest for this study. The methodology used to assess the clinical variables has also been described previously[10].

For the specific purpose of this study, a blood count including hemoglobin, hematocrit, and erythrocytes was conducted using the TOASYSMEX XN-20 device (Roche, Japan).

Patients underwent a complete eye examination to assess the severity of DR and the presence of clinically significant macular edema. Using a modified version of the American Academy of Ophthalmology classification[11], the patients were classified according to the presence or absence of DR, and also to its severity: absence of DR, mild non-proliferative DR, moderate to severe non-proliferative DR, and proliferative DR. For the purpose of this analysis, we selected patients without overt macular edema, and those without DR or with mild non-proliferative DR. We also applied the following exclusion criteria: refractive error (spheric equivalent) greater than ±3 diopters; average opacity that made data analysis difficult; presence of other concomitant retinal pathology; surgery during the previous year; inflammatory conditions in the anterior or posterior segment; treatment for glaucoma; and laser photocoagulation within the previous 6 months. The study was approved by the local Ethics Committee, and all patients gave written informed consent. Serum and spot urine samples were collected in the fasting state, and all serum and urine tests were performed using standard laboratory methods.

Ophthalmic examination and optical coherence tomography

OCT was carried out in all patients to evaluate the macular and peripapillar area[12]. This technology provides quantitative measures of macular thickness, the RNFL and the optic nerve. Using a Stratus Optical Tomography 3 device (Carl Zeiss Meditec, Dublin, CA, USA) under dilated pupil (tropicamide), we applied two distinct OCT protocols in this study:

First, we applied the *Fast RNFL Thickness Protocol*, which estimates retinal and RNFL thickness as the distance between the vitreoretinal interface and the anterior surface of the retinal pigment epithelium and coriocapillar layer. The most prominent feature of the normal peripapillary retinal is a wide red band representing the RNFL. This band is typically wider at the superior and inferior papillary margins and thinner in the temporal and nasal regions. The fast RNFL thickness protocol consists of three circular peripapillary scans of 3.4 mm diameter centred on the optic disc. Each scan consists of 256 measurements along the circumference. We analysed the average overall peripapillary thickness, and the thickness of the superior and inferior quadrants.

Second, we applied the *Fast Macular Thickness Protocol*, which performs 6 linear 6 mm scans focused on the fovea, with a total scan time of 1.92 seconds. Each scans is separated by 30 degrees from the next. The device software fills the gaps between scans by interpolation, and estimates retinal thickness as the distance between the vitreoretinal interface and the union between the internal and external photoreceptor segments located above the retinal pigment

epithelium. The estimated macular thickness is based on a retinal map with nine measures of sectorial thickness in 3 rings centred on the fovea with a diameter of 1, 3 and 6 mm. The areas between the 3 and 6 mm rings and between the 1 and 3 mm rings are the external and internal macular rings, respectively. Each ring is divided into four quadrants: the superior, inferior, temporal and nasal. The retinal map analysis protocol provides the most complete chromatic map of retinal thickness, including a table of macular thicknesses and volumes (in mm and mm^3, respectively) for every internal and external quadrant, the central area and the average value.

Statistical analysis

Normal continuous variables are presented as the mean and standard deviation, and non-normal variables as the median and interquartile range; categorical variables are expressed as proportions. Differences between groups (non-diabetic retinopathy vs. incipient diabetic retinopathy) in anthropometric, clinical, laboratory, hematological and optical coherence tomography variables were evaluated using the chi-square test (for categorical variables), the Mann-Whitney U test (for continuous, non-normal variables), or the Student's t-test (for continuous, normal variables), as appropriate. We evaluated the correlation between continuous laboratory and hematological variables and macular volume and thickness and RNFL thickness. We also tested for correlation between macular thickness and RNFL thickness.

We fit age- and sex-adjusted linear regression models to test whether retinal volume and thickness differed between patients with incipient diabetic retinopathy and those without diabetic retinopathy. We also used these models, further adjusted for intraocular pressure, to ascertain whether the RNFL thickness differed between patients with incipient diabetic retinopathy and those without diabetic retinopathy. The significance level was defined as $p \leq 0.05$, and all analyses were performed using the R v. 3.0[13].

RESULTS

The prevalence of hypertension was significantly higher in patients with mild RD who also presented with significantly higher levels of HbA1c than patients without DR. Hemoglobin and hematocrit levels were higher in individuals with mild DR, but these differences were marginally significant (Table 1).

Patients with mild DR had significantly higher macular volume and thickness (Table 2), although these differences were not significant when the analysis was stratified by quadrant (Figure 1). We observed no significant differences in any of the three RFNL parameters (superior, inferior and mean RFNL) between patients with DR and those without (Table 2). However, RNFL thickness was significantly correlated with age, body mass index and systolic

blood pressure. RFNL thickness was also significantly correlated with hematological factors, namely erythrocyte number, hemoglobin and hematocrit (non-stratified analysis, Table 3). We observed no correlation between macular and RNFL thickness in the full sample (n=216, p-value=0.416).

The sex- and age-adjusted model showed that macular thickness and volume were significantly higher in individuals with mild DR that in patients without DR (Table 4). Additionally, the non-stratified model adjusted for age and intraocular pressure showed a significant association between RNFL thickness and erythrocyte number and hematocrit in the full sample (Table 5).

DISCUSSION

We found that patients at an early stage of DR have greater macular volume and thickness than those without diabetic eye disease, independent of age and sex. We we observed no differences in RFNL thickness between individuals with and without mild DR, this parameter was significantly associated with some hematologic factors: patients with lower erythrocyte numbers and hemoglobin had generally thinner RNFL.

Our results are consistent with previous findings showing increased macular thickness during early DR[5]. This process increases the early vascular permeability that precedes the development of macular edema.

Neurodegeneration has been described as an early event in the pathogenesis of diabetic retinopathy, with available evidence suggesting that overt vascular abnormalities are preceded by neural apoptosis. Several studies reported RNFL thinning in individuals with early DR[1,2,3]. Simo *et al*. have shown that neuronal apoptosis and reactive gliosis are the most important histological findings observed in DR[3]. Retinal ganglion cells located in the internal retina are the first cells to undergo apoptosis, their loss translating into reduced RNFL thickness. While we did not observe any differences in RNFL thickness between patients with and without mild DR, several studies have shown that the RFNL is thinner in individuals with early DR[1,2,7]. One explanation for these results is the inclusion of diabetic subjects without retinopathy as controls, which may make it more difficult to observe differences with respect to cases. It is likely that subtle undetected vascular defects exist prior to or in parallel with the retinal neurodegenerative process in DR, and that these vascular changes may have secondary consequences for neurons[3].

We observed no association between RNFL thickness and glycaemia, but found that age, body mass index and systolic blood pressure were higher in individuals with lower RNFL thickness. Consistent with our results, Shahidi *et al*. showed that type 2 diabetes patients with

no neuropathy had higher RNFL thickness, and lower levels of cardiovascular risk factors, such as body mass index and systolic blood pressure[14].

We also observed that lower erythrocyte numbers and hematocrit, which are indicative of decreased blood oxygen transport capacity, were associated with decreased RNFL thickness in the full patient sample (those without and with mild DR). The retina is very sensitive to reduced oxygen tension because of its high metabolic activity. The development of relative hypoxia as a result of anemia probably stimulates the imbalance in the production of neuroprotective and neurotrophic factors at the retina, such as VEGF and EPO. This process contributes to early neurodegerative disorders in these patients, which is consistent with the observed RNFL thinning.

An increasing body of evidence suggests that neurodegeneration participates in the early microvascular changes that occur in DR[3], including breakdown of the blood–retina barrier[15,16], vasoregression[17], and impaired neurovascular coupling[18,19].Release of inflammation mediators and angiogenic factors, together with neuronal apoptosis and the reactive gliosis, promotes breakdown of the blood–retina barrier. The increasing vascular permeability produces early retinal thinning, which is detectable at early stages of DR, even before the onset of overt macular edema[3]. Our results are consistent with these previous findings. Additionally, vasoregression has been described as the primary response of retinal microvessels to chronic hyperglycemia and is characterized by the loss of pericytes followed by the formation of acellular, non-perfused capillaries[20]. Finally, regarding impaired neurovascular coupling, glial cells have a key role in the hemodynamic response that governs the neurovascular coupling[3].

Limitations

This study has several potential limitations. Its cross-sectional design does not allow us to establish causality. As for any cross-sectional study, this design may also be affected by selection bias, although is not likely to be severe in this study because participants were not selected for the presence or absence of incipient diabetic retinopathy. In addition, we did not include a control group of non-diabetic subjects to compare with the results from diabetic individuals with and without incipient retinopathy. Future studies should compare these parameters with spectral domain OCT (SD-OCT) [21], particularly in non-diabetic subjects and in diabetics with and without mild DR.

CONCLUSION

Diabetes patients with mild DR have higher macular thickness and volume than those without retinopathy. We observed no differences in RFNL thickness between these two patient groups. In addition, RNFL thinning was associated with lower erythrocyte numbers and

hematocrit, independently of the prevalence of DR. This finding highlights the potential role of low blood oxygen transport capacity in the development of retinal microvascular changes and retinal neurodegeneration in diabetes mellitus. Further studies are needed to confirm these results, particularly to compare subjects with diabetes and diabetic retinopathy to the general population.

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Figure 1. Macular volume quadrants in individuals with and without incipient diabetic retinopathy

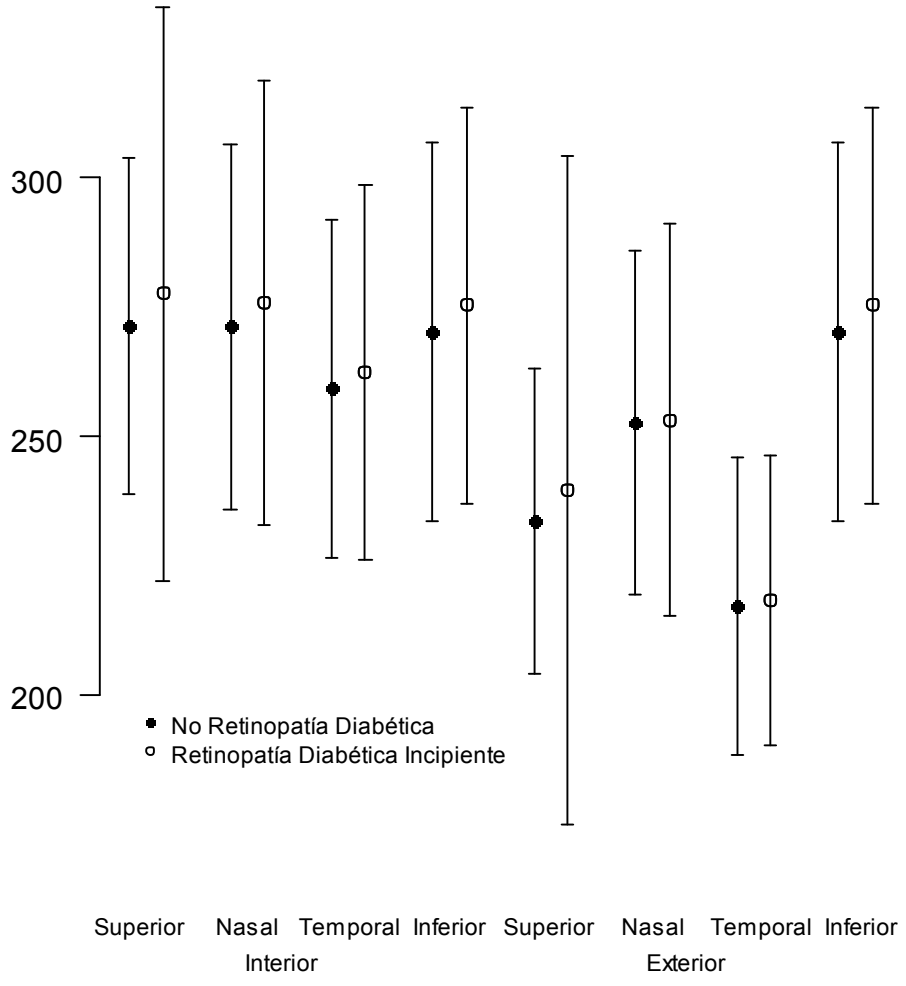

Table 1. Comparison of cardiovascular and hematologic risk factors between individuals with and without mild diabetic retinopathy

	Diabetic Retinopathy		
	No (N=159)	Mild (N=57)	p-value
Sex (women), n (%)	82 (51.6)	26 (54.4)	0.833
Age, mean (SD)	58 (10)	59 (9)	0.253
Hypertension, n (%)	76 (47.8)	38 (66.7)	0.022
Dyslipidemia, n (%)	71 (44.7)	31 (54.4)	0.268
Body mass index, mean (SD)	31.2 (5.2)	31.9 (5.4)	0.449
Waist circumference, mean (SD)	104.0 (11.8)	107.0 (11.9)	0.109
Smoking status, n (%)			0.916
Non-smoker	71 (45.2)	28 (49.1)	
Former smoker	32 (20.4)	12 (21.1)	
Current smoker	54 (34.4)	17 (29.8)	
Systolic blood pressure, mean (SD)	134 (16)	139 (20)	0.142
Diastolic blood pressure, mean (SD)	77 (10)	77 (12)	0.746
Glucose, median [interquartile range]	140 [116-166]	150 [114-181]	0.323
Total cholesterol, mean (SD)	186 (36)	190 (40)	0.491
HDL cholesterol, mean (SD)	48 (11)	50 (15)	0.485
Triglycerides, median [interquartile range]	118 [90-169]	136 [87-179]	0.360
LDL cholesterol, mean (SD)	111 (30)	110 (33)	0.787
High sensitivity C-reactive protein, median [interquartile range]	2.8 [1.4-5.7]	2.2 [1.1-4.7]	0.223
Iron, mean (SD)	81.7 (25.9)	81.8 (25.8)	0.981
Ferritin, median [interquartile range]	131.2 [55.5-227.8]	93.3 [43.2-216.8]	0.250
HbA1c, mean (SD)	7.3 (1.2)	8.1 (1.4)	<0.001
Erythrocytes, mean (SD)	4.7 (0.4)	4.8 (0.4)	0.103
Hemoglobin, mean (SD)	13.8 (1.3)	14.1 (1.1)	0.063
Hematocrit, mean (SD)	41.2 (3.4)	42.1 (3.0)	0.065
Mean corpuscular volume, mean (SD)	87.6 (4.9)	87.7 (4.3)	0.957
Mean corpuscular hemoglobin, mean (SD)	29.3 (1.9)	29.5 (1.9)	0.701

Table 2. Comparison of macular optical coherence tomography and macular parameters between individuals with and without mild diabetic retinopathy

	Diabetic retinopathy		
	No (N=159)	Incipient (N=57)	p-value
Mean macular thickness, mean (SD)	203.2 (22.1)	212.9 (29.1)	0.026
Mean macular volume, mean (SD)	0.16 (0.02)	0.17 (0.02)	0.030
Superior RFNL thickness, mean (SD)	113.6 (15.0)	112.5 (16.3)	0.666
Inferior RFNL thickness, mean (SD)	128.4 (17.9)	124.0 (14.5)	0.072
Mean RFNL thickness, mean (SD)	95.9 (10.5)	95.2 (8.9)	0.647
Visual acuity, mean (SD)	0.84 (0.18)	0.80 (0.19)	0.231
Intraocular pressure, mean (SD)	16.46 (2.21)	16.73 (2.81)	0.510

Table 3. Correlation between macular thickness, macular volume and retinal nervous fiber layer thickness, and cardiovascular and hematological risk factors

	Macular Thickness	p-value	Macular Volume	p-value	RFNL Thickness	p-value
Age	0.18 (0.05; 0.31)	0.007	0.17 (0.04; 0.30)	0.012	-0.28 (-0.40; -0.16)	<0.001
Body mass index	0.01 (-0.13; 0.14)	0.914	0.02 (-0.12; 0.15)	0.789	0.14 (0.00; 0.27)	0.043
Waist circumference	0.03 (-0.10; 0.17)	0.620	0.05 (-0.09; 0.18)	0.493	0.11 (-0.03; 0.24)	0.115
Systolic blood pressure	0.04 (-0.09; 0.17)	0.544	0.05 (-0.08; 0.18)	0.461	-0.16 (-0.29; -0.03)	0.020
Diastolic blood pressure	-0.02 (-0.16; 0.11)	0.749	-0.02 (-0.15; 0.12)	0.792	-0.10 (-0.23; 0.04)	0.152
Glucose	0.00 (-0.13; 0.14)	0.968	0.00 (-0.14; 0.13)	0.980	0.02 (-0.12; 0.15)	0.824
Total cholesterol	-0.11 (-0.24; 0.03)	0.115	-0.12 (-0.25; 0.01)	0.080	0.01 (-0.12; 0.15)	0.858
HDL cholesterol	-0.04 (-0.17; 0.09)	0.545	-0.05 (-0.18; 0.09)	0.490	-0.04 (-0.18; 0.09)	0.536
Triglycerides	0.02 (-0.12; 0.15)	0.816	0.02 (-0.12; 0.15)	0.801	0.09 (-0.04; 0.23)	0.175
LDL cholesterol	-0.11 (-0.24; 0.03)	0.121	-0.12 (-0.25; 0.01)	0.080	-0.04 (-0.17; 0.10)	0.610
High sensitivity C-reactive protein	-0.04 (-0.17; 0.10)	0.569	-0.04 (-0.17; 0.10)	0.581	0.09 (-0.04; 0.22)	0.188
Iron	-0.01 (-0.14; 0.13)	0.924	-0.01 (-0.14; 0.13)	0.916	-0.01 (-0.14; 0.13)	0.905
Ferritin	0.02 (-0.11; 0.16)	0.735	0.02 (-0.11; 0.16)	0.740	-0.04 (-0.18; 0.09)	0.543
HbA1c	0.03 (-0.10; 0.16)	0.658	0.02 (-0.11; 0.16)	0.760	0.00 (-0.13; 0.14)	0.963
Erythrocytes	-0.01 (-0.14; 0.12)	0.891	0.00 (-0.13; 0.14)	0.968	0.21 (0.08; 0.34)	0.002
Hemoglobin	0.01 (-0.13; 0.14)	0.901	0.03 (-0.11; 0.16)	0.671	0.14 (0.01; 0.27)	0.035
Hematocrit	0.01 (-0.12; 0.14)	0.890	0.03 (-0.11; 0.16)	0.673	0.16 (0.02; 0.29)	0.021
Mean corpuscular volume	0.03 (-0.10; 0.16)	0.648	0.04 (-0.10; 0.17)	0.565	-0.09 (-0.22; 0.04)	0.180
Mean corpuscular hemoglobin	0.03 (-0.11; 0.16)	0.698	0.04 (-0.10; 0.17)	0.579	-0.07 (-0.20; 0.06)	0.306

Table 4. Association between mean volume and mean thickness of the macula and diabetic retinopathy

	Mean thickness		Mean volume	
	Beta Coefficient (95% CI)	p-value	Beta Coefficient (95% CI)	p-value
Diabetic retinopathy	0.016 (0.002; 0.029)	0.022	19.837 (2.512; 37.162)	0.025
Age	0.006 (-0.026; 0.039)	0.708	0.007 (-0.026; 0.039)	0.686
Sex	-0.068 (-0.697; 0.562)	0.833	-0.065 (-0.694; 0.565)	0.840

Table 5. Association between RNFL thickness and cardiovascular and hematologic risk factors

	Average RNFL thickness	
	Beta coefficient (95% CI)	p-value
Body mass index	0.197 (-0.053; 0.446)	0.123
Systolic blood pressure	-0.064 (-0.144; 0.016)	0.119
Erythrocytes	4.376 (1.239; 7.513)	0.007
Heamoglobin	0.929 (-0.084; 1.942)	0.074
Heamatocrit	0.430 (0.041; 0.818)	0.031

Models adjusted for age and intraocular pressure

5.3. ARTÍCULO 3

Para evaluar la señal de vasa vasorum carotídeo en pacientes con DM tipo 2, seleccionamos 51 pacientes con RD y 56 sin RD según criterios anteriores. Valoramos también la señal de referencia de VV en un grupo de 65 voluntarios sanos. Se describe por primera vez el método utilizado, siendo el primer estudio en la literatura que utiliza esta metodología. En todos ellos realizamos una ecografía con contraste de microburbujas en un segmento de la pared arterial de la carótida común libre de placa. La utilización de microburbujas nos permitió estudiar las estructuras vasculares, ya que estos compuestos no abandonan el torrente sanguíneo y, por tanto, los VV pudieron ser visualizados y su señal determinada. También evaluamos la presencia o no de contraste en las placas de ateroma, como medida cualitativa de neoangiogénesis intraplaca. Utilizamos la ecografía carotídea sin contraste (Doppler color y modo-B) para medir el GIM e identificar la presencia de placas carotídeas en todos estos pacientes. Correlacionamos estos resultados entre los pacientes con DM y los voluntarios sanos, así como con los subgrupos de pacientes diabéticos, en función de la presencia o no de RD.

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Microangiopathy of large artery wall: A neglected complication of diabetes mellitus

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ABSTRACT

Objective: To test the concept that diabetic patients with microangiopathy of the retinal microcirculation would also show an involvement of the carotid adventitial microcirculation, we aimed to assess the status of the vasa vasorum (VV) signal, measured by contrast-enhanced carotid ultrasound imaging, in type 2 diabetic patients with and without retinopathy.

Methods and results: Using contrast-enhanced ultrasound imaging, we quantified the signal of the VV of the common carotid artery. We investigated two subgroups of type 2 diabetic patients who did not have previous cardiovascular disease: 51 with retinopathy and 56 without retinopathy. The reference VV signal was measured in a group of 65 healthy volunteers as the ratio of the contrast agent signal of the VV and that of the lumen of the artery. Patients and volunteers also underwent a clinical evaluation. The reference VV signal in the group of 65 healthy volunteers was 0.562 (SD = 0.142). Patients with diabetic retinopathy showed a higher mean adventitial VV signal (0.700; SD = 0.150) than those without retinopathy (0.621; SD = 0.120) ($P < 0.0039$). This difference remained highly significant after adjusting for cardiovascular risk factors. Common carotid intima–media thickness and carotid plaque prevalence were not different between diabetic subgroups.

Conclusions: Type 2 diabetic patients with retinopathy show increased angiogenesis of the VV of the common carotid artery. This suggests the existence of a diabetic microangiopathic complication affecting the wall of the large arteries that may be an important contributor to the cardiovascular disease burden in diabetes mellitus.

1. Introduction

Patients with type 2 diabetes mellitus are at increased risk for cardiovascular morbidity and mortality [1]. Additionally, the presence of diabetic retinopathy and its severity are associated with a further increase in the risk of cardiovascular events [2]. A current line of thought suggests that there is a "common soil" for the development of microangiopathic and macroangiopathic complications of diabetes [3]. Indeed, both types of complications share common pathogenetic mechanisms [4–6].

The walls of the large arteries receive their nutrient and oxygen supply from the lumen and also from small vessels located in the adventitia. This plexus of microvessels in the arterial wall of the arteries is named the vasa vasorum (VV) [7]. Barger et al. offered the seminal hypothesis on the role of neoangiogenesis of VV in the pathogenesis of coronary artery disease [8]. Later studies during the 1990s found evidence for a role of intimal neovascularisation originating mainly from the adventitia in the appearance of coronary ischaemic events [9,10]. The main stimuli driving VV angiogenesis are hypoxia and ischaemia [7], a situation analogous to the retinal vascular changes that take place in diabetic retinopathy [11].

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Several authors have suggested that microvascular pathology may contribute to the atherosclerosis progression that occurs in type 2 diabetes and the associated metabolic disturbances [4–6,12]. However, they focused on the contribution of neovascularisation arising from the VV to atherosclerotic plaque progression and rupture, an approach that may demonstrate the interconnection between diabetic microangiopathy and macroangiopathy. We took a step further and hypothesized that large-artery microvessels are a target site for diabetic microangiopathy similar to other well-recognized target microvessels in the retina, the kidney glomerulus, and the peripheral nerve. However, more than four decades ago in a post-mortem study [13], Angervall et al. found that the VV of the aorta of young insulin-treated diabetic patients showed the characteristic features of diabetic microangiopathy. These authors already stated that "the lesions of the vasa vasorum are a conceivably important factor in the development of aortic atherosclerosis".

Chronic hyperglycemia, which characterizes diabetes mellitus, affects the microvascular beds of classical target organs. Thus, we suggest that hyperglycemia originates microangiopathic changes in the VV of large arteries that could explain, at least in part, the differential characteristics of diabetic atherosclerosis. In contrast to the common ground hypothesis, we hypothesize that microangiopathy of the wall of large arteries could then be the substrate where a more severe and rapidly evolving form of atherosclerotic process develops. It is conceivable, according to this hypothesis, that patients already carrying diabetic microangiopathic complications will also have increased VV angiogenesis.

To test the concept that diabetic patients with microangiopathy of the retinal microcirculation would also show an involvement of the carotid adventitial VV, we designed this pilot study to compare the status of the VV signal, measured by contrast-enhanced carotid ultrasound imaging, in type 2 diabetic patients with and without retinopathy. Therefore, the endpoint of this proof-of-concept study was to assess a potential difference in the common carotid vasa vasorum between patients with and those without diabetic retinopathy.

2. Methods

This single-centre prospective cross-sectional study was planned as an initial pilot project on the potential differential status of the carotid VV in type 2 diabetic patients with and without retinopathy as the primary outcome. Because of the lack of previous data on the signal of VV using the methods proposed herein no sample size calculation was feasible. Therefore, in this initial study we planned to include at least 50 patients in each study group.

All diabetic patients were recruited from the outpatient clinic at our centre. Diabetes was classified according to 1999 World Health Organization criteria [14]. Potential candidates were identified from the screening diabetic eye disease program of our centre and from the diabetic retinopathy unit. Thirty-two patients were excluded after the screening visit because of the presence of exclusion criteria (previous cardiovascular disease, impaired renal function, increased urine albumin excretion, or not having type 2 diabetes) and 5 additional patients withdrew from the study after the initial screening visit. Also, 21 diabetic patients had to be excluded because conventional and/or contrast-enhanced ultrasound image acquisition was not feasible for one or both left and right carotid arteries (individual anatomical neck or vessel conditions, presence of a plaque in the common carotid artery, low or undetectable contrast signal and contraindication to contrast administration). Finally, 107 type 2 diabetic patients were included. They also underwent a clinical evaluation that included the variables shown in Table 1. A patient was arbitrarily considered to have previous hypertension or dyslipidemia if the patient was taking medication for the given condition. Weight, height and waist circumference were measured by standardised methods. Blood pressure (mean of 2 measurements 5 min apart) was measured using a blood pressure monitor (HEM-7001E, Omron, Spain) after 10 min in the seated position. Patients underwent a full eye evaluation by one experienced ophthalmologist (AT) to assess the presence or absence of diabetic retinopathy according to an international clinical diabetic retinopathy consensus [15]. Apart from the anamnestic evaluation and physical exam, the clinical records of the patients were reviewed to rule out any previously known cardiovascular events or associated revascularization procedures: coronary heart disease, cerebrovascular disease, and peripheral vascular disease (including the diagnosis of diabetic foot disease). Ours is the only reference hospital for cardiovascular diseases and procedures in the region, but we also had access to reports from the three other small hospitals in the region. Therefore, apart from the

Table 1
Characteristics of the study groups.

	Control ($n = 65$)	Diabetes without retinopathy ($n = 56$)	Diabetes with retinopathy ($n = 51$)	*P* value for group differences	Retinopathy vs. no retinopathy
Sex (male/female)	31/34	29/27	22/29	0.6702	0.3710
Age (yr)	50 (41–57)	58 (49–67)	60 (54–67)	<0.0001	0.1817
Disease duration (yr)	–	5 (2–10)	12 (8–20)	–	<0.0001
Insulin treatment (n)	–	6	32	–	<0.0001
Smoking (current/past/never)	0/2/63	11/19/26	11/19/21	<0.0001	0.8612
Dyslipidemia (yes/no)	0/65	25/31	32/19	<0.0001	0.0609
Hypertension (yes/no)	0/65	19/37	35/16	<0.0001	0.0003
Systolic BP (mm Hg)	125 (115–135)	136 (126–148)	148 (134–162)	<0.0001	0.0025
Diastolic BP (mm Hg)	76 (70–83)	79 (72–84)	80 (70–86)	0.3261	0.8809
Body mass index (kg/m^2)	25.1 (22.9–26.9)	29.7 (26.8–34.7)	30 (27.9–34.7)	<0.0001	0.5852
Waist circumference (cm)	90 (84–98)	99 (92–109)	108 (100–116)	<0.0001	0.0070
Haemoglobin A_{1c} (%)	NA	7.4 (6.7–8.3)	8.4 (7.4–9.1)	–	0.0026
Total cholesterol (mmol/l)	4.83 (4.52–5.3)	4.74 (4.27–5.46)	4.71 (4.07–5.28)	0.6202	0.5185
HDL-cholesterol (mmol/l)	1.37 (1.17–1.61)	1.22 (1.04–1.45)	1.27 (0.98–1.5)	0.1142	0.6465
LDL-cholesterol (mmol/l)	3.08 (2.58–3.48)	2.74 (2.41–3.29)	2.68 (2.11–3.42)	0.0345	0.2682
Triglycerides (mmol/l)	0.72 (0.58–1.03)	1.33 (0.93–2.02)	1.30 (1.01–1.76)	<0.0001	0.7646
Serum creatinine (μmol/l)	73.4 (60.1–84)	69 (61–84.9)	76 (61.9–83.1)	0.7344	0.4172
Urinary albumin:creatinine ratio, mg/g	NA	5.8 (3.3–11.3)	12.6 (5–25.4)	–	0.0014
Fibrinogen (g/l)[a]	NA	3.03 (2.71–3.67)	3.34 (2.93–4.54)	–	0.0376
Haematocrit (%)	NA	41.5 (38.4–43.9)	41.1(38.5–43.1)	–	0.8800

Data are presented as medians (25th and 75th percentiles) or absolute numbers. BP: blood pressure.
[a] Fibrinogen was determined in 54 subjects without retinopathy and 45 subjects with retinopathy.

anamnestic evaluation, we could ascertain any cardiovascular events that occurred in the health-care area.

Blood and spot urine samples were collected in the fasting state. All blood and urine tests were performed using standard laboratory methods. Low-density lipoprotein cholesterol was estimated by the Friedewald formula. Glomerular filtration rate was estimated by the modification of diet in renal disease formula. Haemoglobin A_{1c} (HbA_{1c}) was determined by HPLC (Variant™, Bio-Rad Laboratories S.A., Spain) and its concentrations are expressed in National Glycohemoglobin Standardization Program/Diabetes Control and Complications Trial units. Urine albumin was measured by an immunoturbidimetric method on a Roche/Hitachi Modular P analyzer (Roche Diagnostics, Spain). Plasma fibrinogen was measured by a highly sensitive two-site ELISA assay (Alpco Diagnostics, Salem, NH, USA).

The inclusion criteria for diabetic patients were age 40–70 years, absence of known cardiovascular disease, absence of established impaired renal function (calculated glomerular filtration rate $<$ 60 ml/min) and presence or absence of diabetic retinopathy for subgroup allocation. To avoid including patients with potential additional microangiopathic burden, patients without retinopathy also had a current and previous normal urinary albumin:creatinine ratio ($<$30 mg/g). In the subgroup of diabetic patients with retinopathy, only macroalbuminuria (urine albumin:creatinine ratio $>$ 299 mg/g) was an additional exclusion criterion. We selected patients of several different ages, grouped in 5-year intervals, to achieve a uniform age distribution in diabetic patients.

We studied 65 healthy volunteers to compare their baseline reference VV signal with those of patients suffering various conditions currently under study by our group. The age range for this group was 30–70 years, and they did not have a history of cardiovascular disease or any of the following conditions: diabetes mellitus, renal disease, dyslipidemia, hypertension, or being an active smoker. The control group was also assessed for the same variables, except for haemoglobin A_{1c}, plasma fibrinogen and microalbuminuria.

2.1. Carotid ultrasound imaging study

All study participants underwent the same carotid ultrasound protocol. The assessment of all the measures and performance of ultrasound studies were performed by the same researcher (MVA), who was blinded to the conditions of the participants and did not have access to the study data. VV signal and IMT measurements were done on common carotids excluding segments with plaque. All conventional and contrast-enhanced ultrasound imaging was performed with a Siemens Sequoia 512 using a 15-Mhz linear array probe with a low mechanical index (0.4–1.4).

Conventional ultrasound (B-mode and colour Doppler) was used to measure common carotid intima–media thickness (cIMT) and to identify the presence of carotid plaques according to the Mannheim consensus [16]. All participants underwent a standard ultrasound examination of the extra-cranial carotid arteries (common, bifurcation, internal, and external). After an axial exploration, the carotid arteries were explored longitudinally to evaluate the presence of plaque and to measure the cIMT of the far wall of the common carotid artery at the level of 1 cm proximal to the bifurcation. The cIMT was calculated as previously described [17] as the grey-scale layer contained within the luminal blood and the carotid adventitia layer. For the purpose of this study, we only used the cIMT mean value of both common carotid arteries as the relevant variable, as this was the arterial territory explored for the main outcome of this study. Frequency of carotid plaques was defined as the presence of plaques in any of the explored territories (by study design, none of the participants had a plaque in the common carotid artery).

Subsequently, patients underwent a contrast-enhanced ultrasound imaging. The exclusion criteria for the ultrasound study were those related to contraindications of the use of the contrast agent, those that precluded the acquisition of images of any of the carotid arteries and the presence of a carotid plaque in the common carotid artery. As a contrast agent, we used Sonovue (Bracco Spa, Italy), which consists of a phospholipid shell containing sulphur hexafluoride. A contrast vial was solubilised in 5 ml of saline, and a 2.5 ml bolus, followed by a 10 ml saline flush, was injected into an antecubital vein for each carotid artery explored. The contrast agent at this dose was enough to obtain a strong and clear signal for 1 min of image recording.

The Siemens Sequoia 512 device is equipped with a cadence contrast pulse sequencing software that strengthens the sensitivity and specificity of contrast agent detection. The images were stored for a subsequent reading using Syngo software (Siemens, Germany). For the quantitative measurement of the adventitial VV signal, we developed a reading method. We followed the principle of normalizing the VV to lumen contrast intensity as described by others [18]. Specifically, the intensity of the VV signal measured 1 cm proximal to the bifurcation on the far wall of the common carotid artery, was calculated as the ratio of the intensity in the 2 mm above the intima–lumen boundary and the intensity of the 2 mm below the media–adventitia boundary (Suppl Fig. 1). Since the contrast signal intensity depends on the subject, the ratio was calculated for all the frames where both the lumen and the adventitia layer reached a stable high intensity signal within the 1-min DICOM video acquired during the carotid contrast-enhanced ultrasound examination. The final result was the average of the ratios calculated for each diastolic frame (minimum 10 and maximum 20 frames) of the 1-min DICOM video acquired during the carotid contrast-enhanced ultrasound examination. The intra-observer variability of the VV image reading, calculated through the intraclass correlation coefficient, yielded a value of 0.93 (95% confidence interval: 0.84–0.97). Finally, a qualitative assessment of the presence of contrast in the plaques was performed; only the absence or presence of intraplaque contrast was assessed (yes/no).

The local Ethics Committee approved the protocol, and all patients signed the written informed consent form.

2.2. Statistical analysis

Data are presented as medians and 25th and 75th percentiles, mean $\pm$ standard deviation (SD), and n (%), as appropriate. Group differences (between the two diabetic and the three study groups) in anthropometric, clinical, and laboratory variables were evaluated by the chi-squared test (for categorical variables), Wilcoxon or Kruskal–Wallis test (for continuous non-normally distributed variables), or student's t-test or one-way ANOVA (for continuous normally distributed variables). We investigated the associations between variables of interest and the VV signal. The unpaired Student's t-test was used to evaluate VV signal differences by gender or in patients with vs. without hypertension, dyslipidemia, or smoking habit. Spearman's correlation analysis was used to quantify cross-sectional relationships between continuous variables (e.g., age, lipids, and blood pressure) and the VV signal. Unadjusted and adjusted general linear models (PROC GLM in SAS) tested whether VV signal (or cIMT) differed by group (normal, patients without retinopathy and patients with retinopathy) and between diabetic patients with retinopathy vs. those without retinopathy. Univariate or multiple logistic regression models were used to evaluate the difference in plaque presence between groups.

The significance level was defined as $P \leq 0.05$. Analyses were performed with SAS software, v. 9.2 (SAS Institute Inc., USA).

3. Results

The clinical characteristics of the study groups are shown in Table 1. The sex distribution did not differ between groups. However, as expected, age, cardiovascular risk factors, disease duration, and microalbuminuria were different between groups due to the selection criteria or the metabolic condition and disease stage of the participants. Diabetic patients with retinopathy, compared with those without retinopathy, had higher prevalence of hypertension, systolic blood pressure, waist circumference, HbA_{1c} concentration, urinary albumin excretion, plasma fibrinogen concentration and were more frequently treated with insulin (alone or in combination with oral agents) (Table 1).

The results of the standard and contrast-enhanced carotid ultrasound study are shown in Table 2 (see also Suppl Fig. 2). As a first step, we analysed whether there was an association between the VV signal and any other study variables. In control participants, we found no association between the VV signal and any of the variables. However, in patients with diabetes, disease duration ($P = 0.0068$) and microalbuminuria ($P = 0.0286$), which were both different as predefined selection criteria of each group, were associated with the VV signal. Notwithstanding, we modelled a multiple stepwise regression including the presence/absence of retinopathy, disease duration and microalbuminuria and found that only diabetic retinopathy status was retained as the variable associated to VV signal ($P = 0.0127$). Mean VV signal steadily increased from normal to diabetic patients without and with retinopathy ($P < 0.0001$ for group differences). No association was found between VV signal and markers of blood viscosity and coagulability (haematocrit and fibrinogen, respectively). Additionally, as a group, patients with diabetes compared to control individuals had a higher VV signal (0.658; SD = 0.140; $P < 0.0001$). Amongst diabetic patients, compared with those without retinopathy, those with retinopathy had a higher VV signal both before ($P = 0.0033$) and after ($P = 0.0039$) adjustment for age and sex (Table 2). Although other cardiovascular risk factors (smoking habit, dyslipidemia, hypertension, and systolic blood pressure) were not associated with VV signal intensity, even the adjustment for these variables did not significantly modify the difference between diabetic subgroups ($P = 0.0210$). Even the addition of body mass index, insulin treatment and HbA_{1c} to the model did not produce any changes in the results ($p = 0.0353$).

Concerning the conventional ultrasound measures, cIMT was different between groups ($P < 0.0001$ for group differences) and was not different in diabetic patients with retinopathy compared with those without retinopathy ($P = 0.0828$). These results did not change after adjustment for common well-known cardiovascular risk confounders (Table 2). Furthermore, cIMT was associated with VV signal intensity ($r = 0.18$, $P = 0.0182$), but this association disappeared after adjustment for age ($P = 0.2680$). Finally, carotid plaque prevalence was different between groups ($P < 0.0001$ for group differences) but not between the two subgroups of diabetic patients ($P = 0.0778$). These results did not change after adjustment for well-known cardiovascular risk factors (Table 2). Also, there was no association between the VV signal and the presence of atherosclerotic plaques. The presence of intraplaque neovascularisation was identified in 3 patients with retinopathy and 5 without retinopathy (not significantly different).

4. Discussion

We have shown for the first time that type 2 diabetic patients with retinopathy display an increased VV signal, measured by contrast-enhanced ultrasound imaging, as a sign of increased angiogenesis of the adventitial VV of the common carotid artery wall. Furthermore, compared with patients without retinopathy, even after adjusting for cardiovascular risk factors, this difference remained highly significant. This finding supports our initial hypothesis that neoangiogenesis of the adventitial VV could be an important diabetic microangiopathic complication. Furthermore, although investigating this was not a primary objective of the current study, it is noteworthy that diabetic patients had an increased VV signal when compared with non-diabetic controls. Even in the absence of retinopathy, diabetic patients showed an increased VV signal. This finding deserves further investigation as this suggests that adventitial neoangiogenesis in large arteries may precede the onset of diabetic retinopathy. Although the results only give indirect evidence and indicate an association between retinopathy and VV angiogenesis, the results should encourage further research in this direction.

To our knowledge, this is the first published attempt to quantify the status of adventitial VV in an arterial segment that is free of plaques in healthy volunteers and diabetic patients. Actually, the few studies that previously used contrast-enhanced ultrasound imaging for the assessment of adventitial VV neovascularisation have explored the presence of increased adventitial and/or plaque neovascularisation in carotid plaques. Feinstein and co-workers were the first to use contrast-enhanced ultrasound imaging to study the implication of large-artery microvessel disease in the development of clinical cardiovascular disease [19]. Their studies demonstrated that adventitial VV and plaque neovascularisation are associated with plaque severity and cardiovascular events [20,21]. Additional work using this technique found that the presence of plaque neovessels correlates with histological findings [22,23]. Despite their inclusion of patients with diabetes mellitus,

Table 2
Results of the ultrasonographic carotid study: carotid intima–media thickness, vasa vasorum signal and presence of carotid plaques.

	Control ($n = 65$)	No retinopathy ($n = 56$)	Retinopathy ($n = 51$)	[a]*P* value for group differences	[b]*P* value for retinopathy vs. no retinopathy
VV signal[c]	0.562 (0.142)	0.621 (0.120)	0.700 (0.150)	<0.0001	0.0039
Mean common carotid cIMT (mm)[c]	0.67 (0.13)	0.77 (0.15)	0.82 (0.12)	0.0008	0.6373
Participants with carotid plaques[c]	9 (14)	29 (52)	35 (69)	<0.0001	0.4871

Adjusted *P* values are shown. General linear (for VV signal and cIMT) or multiple logistic regression models (for plaque presence) were used to test group differences.

[a] Because control participants were free of cardiovascular risk factors by design, *P* values were only adjusted for age and sex.

[b] For diabetic subgroup comparisons, variables included in these models were as follows: age and sex for VV signal comparisons; age, sex, smoking habit, dyslipidemia, hypertension, and systolic blood pressure for both cIMT and plaque presence. VV signal and IMT measurements were done on common carotids excluding segments with plaque.

[c] Data presented as mean (SD) or *n* (%).

most of these studies did not provide specific data on diabetic patients. A post-mortem study in young diabetic patients with important cardiovascular burden showed that the characteristic features of diabetic microangiopathy could be identified in the aortic vasa vasorum [13]. Unfortunately, this study had almost no impact on later research into diabetic macroangiopathy. In another post-mortem study, a denser network of VV was found in patients with cardiovascular disease [24]. Moreover, in their patients with cardiovascular disease, including 45% with diabetes mellitus, the hyperplasia of the VV was an early event in symptomatic atherosclerosis. A specific study on plaques from diabetic patients confirmed an increased microvessel content compared with non-diabetic patients [25]. Taking into consideration all these data and the previously suggested involvement of vessel wall microcirculation in the pathogenesis of diabetic atherosclerosis [4–6,12,13], we propose that diabetic microangiopathy of the artery wall microvessels may be the main substrate for specific diabetic macroangiopathic changes. Thus, it is conceivable that there is a response of the microcirculation of the artery wall to ischemia/hypoxia, which also drives microangiopathic changes in other territories like the retina in diabetes mellitus [11]. However, as hyperglycemia is known to adversely affect microvascular and macrovascular beds, further investigation should focus on the study of parallel changes in macro and microcirculation during the progress of atherosclerosis in diabetes mellitus.

Erythrocyte concentration is a primary determinant of blood viscosity that contributes to flow forces at the microcirculatory level [26]. Also, the association of high plasma fibrinogen with diabetic complications, including retinopathy, has been extensively described [27]. The inclusion of markers of viscosity (haematocrit) and coagulation (fibrinogen) in the study of the diabetic patients showed no major contribution of these factors to the difference seen between diabetic patients. Although the number of diabetic subjects included in this study is low, patients with retinopathy showed the expected higher mean plasma fibrinogen concentrations [27].

We did not find differences in terms of cIMT or the number of carotid plaques between diabetic patients with and without retinopathy. If we take into consideration the sample sizes of previous studies assessing cIMT in diabetic retinopathy [28,29], the low number of study participants in the current study prevents us from adopting any firm conclusion concerning these measures of subclinical cardiovascular burden (cIMT and prevalence of plaques); however, these findings may also indicate that the different angiogenic signals detected in the diabetic subgroups represent an early event preceding the advent of atherosclerotic lesions, as suggested by other researchers [6,24]. Recently, Jax suggested that early structural microvascular changes in the arterial wall could mediate the metabolic memory of this target organ [6]. This has important clinical implications, derived from the concept that to prevent the increased cardiovascular morbidity and mortality in diabetes mellitus, we should intervene very early in the course of the disease. Thus, prospective studies are needed to evaluate the pathogenetic sequence of the artery wall microvascular changes in diabetes mellitus.

The limitations of the present study in terms of design and methods must be acknowledged. The study design specifically included a control group that was free of the potential burden of cardiovascular risk factors, and this precludes the assessment of the impact of other factors on the VV signal in non-diabetic patients. Additionally, the lack of an age-matched control group and the limited number of diabetic patients included, together with the absence of previously published data, prevents the extrapolation of these results to the general diabetic population. Therefore, the findings of this study should be tested in future studies with larger sample sizes, especially in patients with type 1 diabetes, who are often free of any other cardiovascular risk factors that contribute to the pathogenesis of atherosclerosis. Following this line of research, based on the current results in type 2 diabetic subjects, a confirmation study in type 1 diabetic patients shall be carried out by our group.

An additional important issue is the method used to study the status of the adventitial microcirculation. We established a method for the assessment of the VV ratio to allow the most objective evaluation of the adventitial signal. Only one experienced researcher carried out the ultrasonographic evaluation and the reading of the images, which yielded high intraobserver reproducibility. The method described will enable its use in future studies in other populations and settings.

The main conclusion of the current study is that type 2 diabetic patients with retinopathy show increased angiogenesis of the VV of the common carotid artery. This finding suggests the potential existence of a diabetic microangiopathic complication affecting the wall of the large arteries. The current findings might be regarded as the result of a proof-of-concept study. Taken as such, this piece of evidence points to the VV of large arteries as a target site for diabetic microangiopathy. This might explain why chronic hyperglycemia leads to a more severe and rapidly evolving form of atherosclerotic disease in diabetes mellitus, with microangiopathy as a substrate of the metabolic memory of large arteries. The available evidence on the role of hyperglycemia as the main etiopathogenic factor of microvascular complications supports the hypothesis that chronic hyperglycemia exerts its specific deleterious effects mainly on large blood vessel wall microcirculation. Future experimental and clinical studies to test the involvement of microvessel wall disease as a chronic microangiopathic specific complication of diabetes mellitus are warranted. This may also have important future preventive and therapeutic implications.

Conflict of interest

All authors declare that there is no conflict of interest associated with this manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.02.011.

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5.4. ARTÍCULO 4

Con el objetivo de valorar el aumento de la carga arteriosclerótica en pacientes con DM tipo 2 y RD, realizamos ecografía carotídea convencional en la arteria carótida común, bulbo y carótida interna. Incluimos en el estudio 153 pacientes con RD y 159 sin RD, según criterios de inclusión antes citados. Recogimos también las características ecográficas de las placas de ateroma, con determinación de los valores de grosor íntima-media (GIM), media y máximo y media global, en los tres territorios estudiados. Correlacionamos estos resultados con la presencia o no de RD.

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Type 2 diabetes-associated carotid plaque burden is increased in patients with retinopathy compared to those without retinopathy

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Abstract

Background: Cardiovascular disease (CVD) is the leading cause of mortality among subjects with type 2 diabetes (T2D), and diabetic retinopathy (DR) has been associated with an increased risk for CVD. The present study was designed to test the concept that T2D patients with DR, but without previous cardiovascular (CV) events and with normal renal function, have an increased atherosclerotic burden compared with patients without DR.

Methods: A cross-sectional study was performed using patients with normal renal function (estimated glomerular filtration rate (eGFR) >60 ml/min) and without previous CV events. A total of 312 patients (men, 51%; mean age, 57 yrs; age range 40–75 yrs) were included in the study; 153 (49%) of the patients had DR. B-mode carotid ultrasound imaging was performed for all of the study subjects to measure the carotid intima-media thickness (cIMT) and carotid plaques in the common carotid artery (CCA), bifurcation and internal carotid artery (ICA).

Results: The percentage of carotid plaques in T2D patients with DR was higher than in T2D patients without DR (68% vs. 52.2%, p = 0.0045), and patients with DR had a higher prevalence of ≥2 carotid plaques (44.4% vs. 21.4%; $p < 0.0001$). No differences were observed in the cIMT measured at different carotid regions between the patients with or without DR. Using multivariate logistic regression (adjustment for major risk factors for atherosclerosis), DR was independently associated with mean-internal cIMT ($p = 0.0176$), with the presence of carotid plaques ($p = 0.0366$) and with carotid plaque burden (≥2 plaques; $p < 0.0001$).

Conclusions: The present study shows that DR in T2D patients without CVD and with normal renal function is associated with a higher atherosclerotic burden (presence and number of plaques) in the carotid arteries. These patients may be at a higher risk for future CV events; therefore, an ultrasound examination of the carotid arteries should be considered in patients with DR for more careful and individualised CV assessment and follow-up.

Keywords: Type 2 diabetes, Cardiovascular disease, Retinopathy, Carotid plaque

Background

The deleterious effects of hyperglycaemia are classically separated into microvascular (retinopathy, diabetic nephropathy and neuropathy) and macrovascular complications (coronary artery disease, peripheral arterial disease and cerebrovascular disease).

Traditionally, the micro- and macrovascular complications of diabetes have been viewed, studied, and treated as distinct and independent disorders. However, accumulating data from epidemiological and pathophysiological studies suggest that these vascular complications may share common pathophysiological mechanisms beyond those related to traditional cardiovascular (CV) risk factors. Data obtained from epidemiological studies have clearly demonstrated that diabetic retinopathy (DR), a common chronic microvascular complication of diabetes, is associated with macrovascular disease [1] as

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well as with increased CV morbidity and mortality in patients with type 2 diabetes mellitus (T2D) [2]. In these patients, the presence of DR has been described as an independent risk factor for incident coronary heart disease [3,4] and ischaemic stroke [5]. Among the possible mechanisms described to explain the interconnection between diabetic micro- and macroangiopathy are metabolic disturbances [6]. Other authors have suggested that microangiopathy of the vasa vasorum (VV), the plexus of microvessels that partially provides oxygen and nutrients to the walls of large arteries, may be involved in diabetic atherosclerosis [7,8]. Our group recently reported that T2D patients with DR had increased angiogenesis of the VV of the common carotid artery (CCA) [9]. These findings were recently confirmed by Sampson *et al.* in a larger study of T2D patients [10].

When a clinical CV event occurs, atherosclerotic disease is difficult to reverse. In these cases, ultrasonography of the carotid arteries is frequently used to detect early signs of atherosclerosis, i.e., increased thickness of the arterial wall or the occurrence of plaques. Ample data suggest that plaque and carotid intima-media thickness (cIMT) are associated with prevalent and incident coronary heart disease (CHD) and stroke, with the presence of plaque generally having a stronger association with CVD compared to cIMT alone [11]. Thus, it has recently been demonstrated that ultrasound assessment of a carotid plaque and its total volume or total area progression is a stronger predictor of future CV events than cIMT measurement [12,13]. A high prevalence (between 43% and 64%) of carotid plaques has been described in T2D patients without evidence of CV disease (CVD) [14-16]. Prospective studies conducted in T2D patients free of any CV event have shown that the percentage of patients with carotid artery plaques is higher in those who develop a CV event compared with those who are free from CV events [15,16]. Recent epidemiological studies have demonstrated that chronic kidney disease (CKD), defined as an estimated glomerular filtration rate (eGFR) <60 ml/min, is an independent risk factor for atherosclerotic disease [17] and for the presence of carotid plaques [18]. In T2D patients, a high prevalence of low eGFR without associated albuminuria has been reported and described to be associated with atherosclerosis of peripheral arteries, independently of albuminuria, [19] and with cIMT in several [20], but not all, studies [21,22].

To test the concept that patients with T2D and DR have an increased prevalence of subclinical atherosclerosis, as shown using carotid plaque burden and cIMT measurements, we investigated the association between these measures and retinopathy in a group of patients with T2D but without previous CVD and with normal renal function.

Methods

Subjects

T2D patients were selected to participate in this single-centre study, which aimed to investigate the association between carotid atherosclerosis and DR in T2D. All study participants were recruited from the outpatient clinic at our centre, and potential candidates were identified by screening patients enrolled in the diabetic eye disease programme of our centre as well as those visiting the diabetic retinopathy unit. The inclusion criteria for both groups were as follows: age range, 40–75 yrs; absence of established impaired renal function (calculated glomerular filtration rate (eGFR) <60 ml/min); and known CVD. From the total number of subjects who were initially recruited, 60 subjects were excluded after the screening visit because of the presence of an exclusion criterion (previous CVD, impaired renal function, increased urine albumin excretion, or not having T2D), and 15 additional patients withdrew from the study after the initial screening visit (consented withdrawal before completing the study assessment). A total of 312 participants with (n = 153) or without (n = 159) DR were included in the study. To achieve a uniform age distribution, patients with and without DR were selected to have representative and similar numbers of patients by gender and age (stratified according to 5-yr age intervals). However, we could not recruit enough patients with retinopathy in the age range between 40 and 50 yrs. For the purpose of this study, a patient was arbitrarily considered to have previous hypertension or dyslipidaemia if the patient was taking medication for the given condition. The weight, height and waist circumference of the subjects were measured using standardised methods, and the blood pressure (mean of 2 measurements, 5 min apart) of the subjects was measured after 10 min in the seated position using a blood pressure monitor (HEM-7001E, Omron, Barcelona, Spain). The patients underwent a complete eye evaluation by experienced ophthalmologists (AT and CJ) to assess the presence or absence of diabetic retinopathy according to an international clinical DR consensus [23]. Retinopathy was evaluated using multifield stereoscopic retinal photography with the following definitions: a) mild nonproliferative DR - microaneurysms only; b) moderate nonproliferative DR - more than just microaneurysms but less than severe nonproliferative DR; c) severe nonproliferative DR by any of the following - more than 20 intraretinal haemorrhages in each of 4 quadrants, definite venous beading in 2+ quadrants, prominent intraretinal microvascular abnormalities in 1+ quadrant, and no signs of proliferative retinopathy; and d) proliferative DR, by neovascularisation and/or vitreous/preretinal haemorrhage. In addition to the anamnestic evaluation and physical examination, the patients' clinical records were reviewed to rule out

any previously known CV events or associated revascularisation procedures, including coronary heart disease, cerebrovascular disease, or peripheral vascular disease (including the diagnosis of diabetic foot disease). Ours is the only reference hospital for CVDs and procedures in the region, but we also had access to reports from three small hospitals in the region. Therefore, along with the anamnestic evaluation, we could ascertain any CV events that occurred in the health-care area. Heart failure and macroalbuminuria (urine albumin/creatinine ratio >299 mg/g) were also considered exclusion criteria. Serum and spot urine samples were collected in the fasting state, and all serum and urine tests were performed using standard laboratory methods. Low-density lipoprotein cholesterol was estimated using the Friedewald formula, and eGFR was estimated using the diet modification in renal disease (MDRD-4) formula [24]. Haemoglobin A_{1c} (HbA_{1c}) levels were determined using HPLC (Variant^{TM}, Bio-Rad Laboratories SA, Spain), and its concentrations are expressed in National Glycohemoglobin Standardization Program/Diabetes Control and Complications Trial units. Urine albumin was measured using an immunoturbidimetric method and a Roche/Hitachi Modular P analyser (Roche Diagnostics, Spain).

Carotid ultrasound imaging study

All of the study participants underwent the same carotid ultrasound protocol. All measures and ultrasound studies were assessed by the same researcher (ER), who was blinded to the conditions of the participants and did not have access to the study data. B-mode ultrasound imaging was performed using a Siemens Sequoia 512 and a 15-Mhz linear array probe. Moreover, a standardised imaging protocol was performed to evaluate intima-media-adventitia thickness (IMT), defined as the distance between the lumen-intima and the media-adventitia ultrasound interfaces (intima-media complex), and plaque presence in the carotid arteries. The patients were examined from their back; they were placed in the supine position with the head turned 45° contralateral to the side of scanning. Images were obtained in longitudinal sections, with a single lateral angle of insonation and optimisation of the image to the far wall. The B-mode images of the left and right segments were recorded and electronically stored. The last (previous to the bulb) and first (starting at the flow divider) centimetres of the common and internal carotid arteries, respectively, and the total length of the bulb were used for IMT measurements, which were performed off-line using semiautomatic software. The data (mean IMT and mean-maximum IMT) from each segment were provided, and the right- and left-side values were averaged to obtain the mean and mean-maximum common carotid (CC), carotid bulb, and internal carotid (ICA) measurements. Plaques were identified using B-mode and colour Doppler examinations in both the longitudinal and transverse planes to consider circumferential asymmetry and were defined as a "focal structure that encroaches into the arterial lumen of at least 0.5 mm or 50% of the surrounding cIMT value or demonstrates a thickness of 1.5 mm, as measured from the media adventitia interference to the intima-lumen surface" according to the Mannheim consensus [25].

The local Ethics Committee of Hospital Arnau de Vilanova (Lleida, Spain) approved the protocol, and all of the patients signed written informed consent forms.

Sample size

To determine the sample size, we used preliminary data from a previous study of our group in which the plaque frequency was 69% in patients with retinopathy or 52% in patients without retinopathy [10]. We calculated a sample size of 126 subjects for each study group, which would allow an 80% power to detect differences between groups with a significance level of <0.05. Thus, the number of patients included in each group was sufficient to test the initial hypothesis.

Statistical analysis

The data are presented as the median values and 25th and 75th percentiles, means ± standard deviations (SDs), and n (%), as appropriate. Non-normally distributed variables were log transformed to reduce skewness, and normality was re-evaluated. Between-group (DR vs. non-DR, male vs. female, and with hypertension vs. without hypertension, or dyslipidaemia, or smoking habit) differences in anthropometric, clinical, cIMT, and laboratory variables were evaluated using the chi-squared test (for categorical variables), Wilcoxon test (for continuous, non-normally distributed variables), or Student's *t*-test (for continuous, normally distributed variables). Unadjusted and adjusted general linear models (PROC GLM in SAS) were used to test whether cIMT values differed between diabetic patients with retinopathy vs. those without retinopathy. Univariate or multiple logistic regression models were used to evaluate the differences in plaque presence between the groups. The following factors were entered into the adjusted general linear and multiple logistic regression models: age, sex, body mass index, presence of hypertension, presence of dyslipidaemia, smoking habit, urinary albumin excretion rate and eGFR. The significance level was defined as $p \leq 0.05$, and all analyses were performed using SAS software, v. 9.2 (SAS Institute Inc., USA).

Results

Clinical variables

Among the 312 study patients with T2D, 153 (49.03%) had DR. The patients with retinopathy were older due to

difficulties recruiting patients with DR who were aged <50 yrs. As typically observed in previous studies comparing patients with and without DR, the patients with this condition had longer diabetes duration and were more likely to receive insulin treatment and antiplatelet agents. These patients also exhibited a higher prevalence of hypertension, higher systolic blood pressure, and increased waist circumference, HbA1c level, HDL-c level, and urinary albumin/creatinine ratio compared with those without DR (Table 1). The distribution of retinopathy grades was as follows: 60 patients had mild retinopathy (39.2%), 57 patients had moderate retinopathy (37.3%), 29 patients had severe nonproliferative retinopathy (18.9%), and 7 patients had severe proliferative retinopathy (4.6%).

Ultrasound examination

The mean and mean-maximum cIMT at different territories (common, bulb, and internal carotid) did not differ between the patients with DR and those without DR, except that a higher mean-maximum common cIMT was observed in the patients with DR ($p = 0.0187$); these differences disappeared after adjustment (Table 2). The general linear models were used to evaluate the cIMT determinants. Age ($p < 0.0001$) and active smoking ($p = 0.0641$); age ($p = 0.0154$) and hypertension ($p = 0.0140$); and age ($p = 0.0131$), sex ($p = 0.0327$), and the presence of retinopathy ($p = 0.0176$) were independently associated with mean CCA-IMT, bulb-IMT, and ICA-IMT, respectively. No association was found between any of the cIMT measurements at different territories or between the UAE and eGFR values or diabetes duration.

The percentage of patients with carotid plaques was higher in those with DR compared to those without DR (68% vs. 52.2%, p = 0.0045), and of these patients, the DR group contained more patients with ≥2 carotid plaques (44.4% vs. 21.4%, $p < 0.0001$). No association was found between carotid plaque burden (≥2 carotid plaques) and the duration of diabetes. In relation to the territory, there was a tendency towards a higher frequency of plaques at the internal carotid artery (ICA) in the group of patients with DR (36% vs. 49%, p = 0.07). The variables that were independently associated with the presence of carotid plaques were age (p = 0.0022), hypertension (p = 0.0300), the presence of retinopathy (p = 0.0366), and active smoking (p = 0.0109) (Table 3). The variables that were associated with the presence of ≥2 carotid plaques were age (p < 0.0001), sex (p = 0.0015), dyslipidaemia (p = 0.0031), and the presence of retinopathy (p < 0.0001) (Table 4).

Table 1 Characteristics of the study groups

	Diabetes without retinopathy (n = 159)	Diabetes with retinopathy (n = 153)	*p*
Sex (male/female)	82/77	76/77	0.7373
Non-Caucasian	7 (4.4%)	6 (4.6%)	0.9413
Age (yr)	59 (48–66)	61 (54–68)	0.0127
Disease duration (yr)	6 (2–10)	11 (6–20)	<0.0001
Insulin treatment (n)	20 (12.6%)	84 (54.9%)	<0.0001
Smoking (current/past/never)	71/54/32	78/44/30	0.5134
Antiplatelet agents	112/47 (29.6%)	82/71 (46.4%)	0.0022
Dyslipidaemia (yes/no)	88/71 (44.7%)	79/74 (48.4%)	0.5111
Hypertension (yes/no)	83/76 (47.8%)	48/105 (68.3%)	0.0002
Systolic BP (mm Hg)	134 (123–145)	143 (133–159)	<0.0001
Diastolic BP (mm Hg)	76 (70–83)	77.5 (68.5-85.5)	0.7526
HR (b/min)	75 (68–82)	77 (70–86)	0.0767
Body mass index (kg/m^2)	30.3 (27.4-34.0)	31.1 (28.3-35)	0.2477
Waist circumference (cm)	103 (96–111)	107.5 (163–114)	0.0039
Haemoglobin A_{1c} (%)	7.1 (6.5-7.9)	8.1 (7.2-9.1)	<0.0001
Total cholesterol (mmol/l)	184 (163–207)	181 (162–204.5)	0.9889
HDL-cholesterol (mmol/l)	47 (40–57)	50.5 (42–60.5)	0.0308
LDL-cholesterol (mmol/l)	108 (90.2-130.2)	105.4 (86.5-127.8)	0.2694
Triglycerides (mmol/l)	118 (89–171)	116 (83–168)	0.6181
Serum creatinine (μmol/l)	0.79 (0.69-0.92)	0.79 (0.68-0.93)	0.7975
Urinary albumin/creatinine ratio, mg/g	5.8 (3.2-11)	12.4 (6–32.7)	<0.0001

Data are presented as the median values and interquartile ranges. BP, blood pressure.

Table 2 Results of the ultrasonographic carotid study: carotid intima-media thickness and presence of carotid plaques

	No retinopathy (n = 159)	Retinopathy (n = 153)	P value
Mean common cIMT	0.789 (0.686-0.889)	0.802 (0.731-0.907)	0.1474
Mean-max common cIMT	0.941 (0.820-1.049)	0.986 (0.889-1.073)	0.0187
Mean bulb cIMT	0.835 (0759–0.955)	0.861 (0.723-0.928)	0.6674
Mean-max bulb	1.047 (0.922-1.199)	1.007 (0.919-1.227)	0.7470
Mean-internal cIMT	0.677 (0-566-0.782)	0.623 (0.534-0.713)	0.1151
Mean-max internal cIMT	0.845 (0.704-1.009)	0.810 (0.685-0.909)	0.2070
Carotid plaques, n (%)	83 (52.2%)	104 (68%)	0.0045
Carotid plaques (none/1/>1) (n)	76/49/34	49/36/68	<0.0001
Common carotid plaque, n (%)	9 (10.8%)	19 (18.3%)	0.1574
Bulb plaque, n (%)	67 (80.7%)	89 (85.6%)	0.3752
Internal carotid plaque, n (%)	30 (36.1%)	51 (49%)	0.0771

No differences in cIMT measurements or in plaque presence were observed among the groups with different grades of retinopathy (mild vs. moderate/severe/proliferative).

Discussion

The present study shows that T2D patients with DR who are free of clinical CVD and with normal renal function have increased atherosclerotic burden in their carotid arteries compared with patients without DR.

Epidemiological studies have demonstrated that DR in T2D patients is associated with a 1.7-fold increased risk of CV events, such as stroke, coronary artery disease and heart failure [26]. Notably, this risk persists after adjusting for traditional CV risk factors, diabetes duration and diabetes control, suggesting that microvascular disease may contribute to the development of CVD in diabetes. A major finding of the present study is that DR is independently associated with the presence of carotid plaques. This result is similar to that observed in the study by Kreutzenberg *et al.*, in which DR was independently associated with carotid plaques, although the number of patients with retinopathy was lower in that study than in our study, and patients with previous CV events were not excluded [27]. On the other hand, several studies have described an association between cIMT thickness, as a surrogate marker of atherosclerosis, and DR that is independent of traditional risk factors and glucose control [28,29]. Miyamoto *et al.* also reported an association between common carotid arterial diameter, as a marker of atherosclerosis, and the presence of DR [30]. It should be noted that in the present study, the percentage of patients with carotid plaques was not only higher in those with DR than in those without DR but that the number of carotid plaques was also elevated. To the best of our knowledge, there is no report in the literature regarding atherosclerotic plaque burden in T2D patients with or without DR. A direct association has been described between a plaque score, consisting of the sum of the maximum plaque thicknesses of different carotid territories, and the presence and extent of coronary artery disease in T2D patients [31]. As reported in general-population studies, the risk

Table 3 Multivariate logistic regression for the presence of carotid plaques

	Odds ratio (95% confidence interval)	P value
Age	1.04 (1.01-1.07)	0.0022
BMI	0.98 (0.93-1.03)	0.4578
Female sex	0.79 (0.47-1.34)	0.3952
DR	1.71 (1.03-2.85)	0.0366
Hypertension	1.78 (1.05-3.00)	0.0300
Dyslipidaemia	1.16 (0.71-1.89)	0.5505
Smoking	2.17 (1.13-4.14)	0.0109
eGFR	1.00 (0.99-1.01)	0.3345
UAE	0.99 (0.99-1.00)	0.6397

DR: diabetic retinopathy; UAE: urinary albumin excretion rate; eGFR: estimated glomerular filtration rate.

Table 4 Multivariate logistic regression for carotid plaque burden (≥2 plaques)

	Odds ratio (95% confidence interval)	P value
Age	1.09 (1.05-1.13)	<0.0001
BMI	0.98 (0.92-1.04)	0.6012
Female sex	0.36 (0.19-0.67)	0.0015
DR	3.17 (1.75-5.75)	<0.0001
Hypertension	2.00 (1.08-3.70)	0.0255
Dyslipidaemia	2.32 (1.33-4.05)	0.0031
Smoking	1.62 (0.77-3.39)	0.1980
eGFR	1.00 (0.99-1.02)	0.3177
UAE	0.99 (0.98-1.00)	0.3107

DR: diabetic retinopathy; UAE: urinary albumin excretion rate; eGFR: estimated glomerular filtration rate.

of CV events, such as stroke and cerebral infarction, gradually increases with incremental total plaque number, irrespective of the location of the plaques [32]. Moreover, carotid plaque measurements have been shown to be more strongly predictive of CV events than cIMT measurements. The extent of atherosclerosis in the carotid artery, measured either as total plaque area [30] or as progression of total plaque volume [12], has been shown to be an independent predictor of coronary events in patients with [12] and without previous CV events [33]. Although these studies included a small proportion of subjects with diabetes, no specific data on these subjects were provided. In the Rotterdam study, a direct relationship between the number of carotid territories with a carotid plaque and incident cases of myocardial infarction was described [34].

Carotid plaque predominantly occurs at sites of non-laminar turbulent flow, such as the carotid bulb and the proximal ICA, but rarely in the CCA, except in advanced atherosclerotic disease [35]. In the present study, the percentage of plaques in the ICA was higher in T2D patients with DR than in those without DR; however, these differences were not significant. The presence of plaques in the ICA territory is now accepted to be associated with an increased risk of stroke (annual risk of 0.1% to 1.9% for an ICA stenosis <75% to 80%, and annual risk of 2.0% to 3% with greater degrees of stenosis) [36]. Thus, considering research results suggesting that CV events increase as the burden of atherosclerosis increases, we hypothesise that T2D patients with DR and >1 carotid plaque will have a higher risk of CV events. This speculation can only be answered by conducting a prospective study assessing the future occurrence of CV events in these patients.

As stated in almost all studies on DR, the condition is closely related to duration of the disease, higher blood pressure and a higher albumin excretion rate, all of which may contribute to the increased atherosclerosis in these patients [37]. In the present study, these factors were also more frequent in patients with DR. However, of these variables, only hypertension proved to be independently associated with carotid plaques.

It is now accepted that CKD, defined as an eGFR of <60 ml/min, is an independent risk factor for atherosclerotic diseases and is associated with the presence of a carotid plaque independent of albuminuria [38]. Recent studies have emphasised that a large number of T2D patients with low eGFR have a normal UAE [39,40]. It should be noted that little information regarding renal function has been reported in those studies that analysed subclinical atherosclerosis in patients with T2D. In this respect, the study by Saif *et al.*, conducted inT2D patients with normal renal function, did describe a correlation between DR and cIMT [29].

Another ultrasound measure that is used as a surrogate marker for atherosclerosis is cIMT. This measure, as described for carotid plaques, has been reported to be associated with traditional and newer risk factors for atherosclerosis. The cIMT baseline measures and its progression during follow-up are described to be strongly associated with CVD events, even after adjustment for traditional risk factors [41]. cIMT has typically been measured in the CCA, rather than in the carotid bulb or ICA, because high-level measurement precision is easily obtained from this artery. However, it should be noted that the accuracy of cIMT as a marker of atherosclerosis has been questioned by the fact that medial hypertrophy or intimal thickening of CCA may be influenced by factors that do not necessarily reflect the atherosclerotic process, such as age and hypertension [42]. In the present study, T2D patients with DR had a higher mean-maximum common cIMT than did those without DR, whereas no differences were observed in the bulb-cIMT or in the internal cIMT measures between the groups. However, DR was independently associated with only the mean-internal cIMT. In relation to cIMT, it is accepted that, clinically, the measurement of CCA-cIMT is easier and more reliable compared to ICA-cIMT. In contrast, ICA-cIMT appears to predict atherosclerotic cardiac events better than common cIMT [43]. Thus, including internal cIMT and traditional CV risk factors, as well as the presence of plaque in the internal territory, improves the risk classifications of stroke and coronary heart disease in general population studies.

The association between DR and carotid ultrasound measurements has been analysed in large-population cohort studies conducted on subjects without a history of clinical CVD. The ARIC study found that the severity of retinopathy was associated with carotid artery intima-media wall thickness [44], while the CHS [45] and MESA [46] studies found no association between DR and cIMT or carotid stenosis. Possible explanations for the different results obtained in these last two studies and ours may be due to the difference in ethnic backgrounds of patient groups, different criteria used for the definition of subclinical atherosclerosis, different population selection or the absence of bulb territory (a site with a high prevalence of plaques) in the ultrasound examination in these studies. In the present study, no differences in cIMT measurements or in plaque presence were observed among the groups with different grades of retinopathy. This finding is in contrast to the findings of Kreutzenberg *et al.* [27], who described that the retinopathy stage was associated with carotid atherosclerosis. Additionally, in the ARIC study, the severity of retinopathy was associated with cIMT. However, in contrast to the present study, patients with previous CV events were not excluded in those studies. Moreover, the

absence of differences observed in cIMT measurements or in plaque presence in relation to retinopathy grades in the present study may be due to few patients in the group having more severe retinopathy. Thus, any conclusion drawn from these results must be made with caution.

In relation to retinopathy and the future risk of CV events, retinopathy has been described in population-based samples as a risk factor (independent from diabetes) for stroke [47], heart failure [48] and coronary heart disease [49]. Patients from the ARIC study who had better CV health, according to the American Heart Association criteria, had a lower prevalence of retinopathy [50]. Moreover, the presence of retinopathy and retinal microvascular signs has recently been described to be associated with an increased risk of cerebral microvascular disease [51].

The strength of the present study is its specific design, which aimed to test the hypothesis that T2D patients with retinopathy have a higher atherosclerotic burden compared with T2D patients without retinopathy. Thus, a large number of patients with retinopathy were included. Only those patients with an eGFR >60 ml/min were included to avoid the confounding factor of renal insufficiency, which is associated with an increased risk of atherosclerosis.

The major limitation of the present study is that a direct relationship between retinopathy and macrovascular disease cannot be demonstrated due to the cross-sectional nature of the study. To assess the CV risk associated with a microvascular complication, such as DR in patients with T2D, longitudinal studies are warranted. To properly assess the CV risk, future studies should include T2D patients with DR (with and without carotid plaques) and T2D patients without DR and other microvascular complications.

Conclusions

The present study, which has been specifically designed to test the hypothesis that T2D patients with DR who are free of previous CVD and have normal renal function have a higher atherosclerotic burden than those without DR, shows an association between DR and subclinical carotid atherosclerosis. More specifically, T2D patients with DR have an increased atherosclerotic burden (presence of plaques and number of plaques) in the carotid territory. Previous studies performed on the general population have shown that individuals with an increased atherosclerotic burden have a higher risk of CV events (myocardial infarction and stroke). Given the increased atherosclerotic plaque burden observed in the T2D patients with DR in the present study, we believe that these patients represent a target population in which ultrasound examination of the carotid artery should be performed to evaluate the presence/absence of atherosclerotic lesions in this location. In our opinion, given the association between the atherosclerotic plaque burden and the future risk of CV events shown in general-population studies, a more careful CV assessment and follow-up should be considered in patients with carotid plaques at ultrasound examination.

Abbreviations

CCA: Common carotid artery; cIMT: carotid intima-media thickness; CKD: Chronic kidney disease; CV: Cardiovascular; CVD: Cardiovascular disease; DR: Diabetic retinopathy; eGFR: Estimated glomerular filtration rate; ICA: Internal carotid artery; VV: Vasa vasorum.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NA wrote and drafted the manuscript. AT and CJ performed the ophthalmological examination. All of the authors, except for DM, EF and EO, contributed to the data collection. EO participated in the study design, performed the statistical analysis and reviewed the manuscript. ER performed all ultrasound examinations. EF and DM conceived the study, participated in its design and coordination and reviewed the manuscript. All of the authors contributed to the discussion and approved the final manuscript.

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6. DISCUSIÓN

La RD se ha sugerido como un marcador de daño microvascular generalizado en los órganos diana de los paciente con DM tipo 2. Su presencia se asocia con un aumento del riesgo de complicaciones vasculares sistémicas potencialmente mortales, incluyendo el accidente cerebrovascular, la insuficiencia cardíaca y la enfermedad cardíaca coronaria.

1. Papel de la baja capacidad transportadora de oxígeno de la sangre en los cambios microvasculares y neuroretinianos observados en pacientes con DM tipo 2.

A pesar de que los mecanismos patogéneticos que conducen a la aparición de la RD no son del todo conocidos, existe un gran número de estudios multicéntricos[64-69] que valoran los factores de riesgo involucrados en su aparición y progresión, siendo la duración de la diabetes, el control glucémico y la presión arterial sistólica los más aceptados y ampliamente documentados. La anemia se ha sugerido como complicación a largo plazo de la DM y, junto con la isquemia, se ha asociado también con el desarrollo y progresión de las complicaciones tanto micro como macrovasculares[70]. Estudios recientes demuestran que no sólo los cambios vasculares sino también las alteraciones neuronales, incluyendo la muerte de células ganglionares, acompañan los cambios patogénicos observados en etapas precoces de la RD[26].

Varios autores proponen que las concentraciones de hemoglobina (Hb), hematocrito y viscosidad sanguínea pueden contribuir al desarrollo y a la progresión de la retinopatía diabética. La presencia de concentraciones bajas de Hb se han considerado como factor de riesgo independiente para el desarrollo de RDP y de pérdida visual grave[19]. Otros estudios han corroborado este hecho y han encontrado una mejoría del grado de RD con la corrección de la anemia[20,21-23].

Los resultados de nuestro estudio no lograron demostrar una asociación independiente entre los niveles de Hb y la presencia de RD, pero sí demostraron que las concentraciones bajas de Hb se asocian con formas más avanzadas de RD y con la presencia, hasta ahora no descrita, de isquemia retiniana. La exclusión específica de otras complicaciones asociadas a la DM, especialmente la nefropatía diabética, nos proporcionó una población de estudio libre de enfermedad micro y macrovascular a excepción de la RD y, por lo tanto, nos evitó el potencial efecto de confusión producido por la influencia de estos factores en las variables principales de los estudios.

Los niveles bajos de hematocrito y eritrocitos se correlacionaron también con un descenso en la capa de fibras nerviosas de la retina en aquellos pacientes sin RD o con RD leve. El estudio de grosor y volumen macular evidenció un engrosamiento mayor en los pacientes diabéticos con RD leve respecto a aquellos sin RD, no observándose diferencias

en el espesor de la capa de fibras nerviosas de la retina (CFNR) entre ambos grupos.

Por ello, si consideramos la anemia como un indicador de menor capacidad de transporte de oxígeno en la sangre, y tenemos presente que la retina es un tejido muy sensible a las reducciones en la presión de oxígeno por su gran actividad metabólica, podemos apuntar que una menor concentración de Hb en pacientes diabéticos puede favorecer la hipoxia e isquemia retiniana y, con ello, contribuir a las alteraciones microvasculares y neuroretinianas observadas en estos pacientes.

La isquemia retiniana favorece la disregulación de factores neuroprotectores y neurotróficos, tales como la EPO y el VEGF. La sobreexpresión de estos factores contribuye a las alteraciones neurodegenerativas precoces detectadas en pacientes diabéticos. La liberación de mediadores inflamatorios y de estos factores angiogénicos contribuye también, junto con la apoptosis neuronal y la gliosis reactiva, a la alteración de la BHR y a la vasoregresión que observamos en los pacientes con RD. El aumento de la permeabilidad vascular es capaz de producir un engrosamiento retiniano precoz, ya detectable en etapas iniciales de la RD, antes de la aparición del edema macular clínicamente establecido. Esto nos permitiría explicar por qué las concentraciones bajas de hemoglobina se asocian con un aumento en la gravedad de la RD y con la presencia de isquemia retiniana, contribuyendo así al desarrollo y/o a la progresión de la retinopatía diabética.

2. Interrelación entre la micro y la macroangiopatía en la arteriosclerosis de pacientes diabéticos.

En la diabetes mellitus tipo 2, la aterotrombosis acelerada es un proceso frecuente que contribuye al aumento de la morbilidad y mortalidad cardiovascular. La angiogénesis de los VV de la adventicia arterial y el engrosamiento del complejo íntima-media arterial constituyen cambios precoces en el desarrollo de la placa de ateroma de estos pacientes.

En nuestro grupo de estudio, observamos un aumento de la señal de VV carotídeo en aquellos pacientes diabéticos con RD respecto a aquellos sin RD, como signo indirecto de un incremento de la angiogénesis en la adventicia de la pared de la arteria carótida. Esta asociación se mantuvo después de ajustar por factores, incluidos todos los de riesgo cardiovascular. Los pacientes diabéticos respecto a los controles sanos, también mostraron un aumento de la señal de VV, incluso en ausencia de RD. Este hallazgo apoya la hipótesis de que la neoangiogénesis de los VV de la adventicia arterial puede ser un indicador de la existencia de una complicación microangiopática de la diabetes que afecta la pared arterial, precediendo incluso a la aparición de la RD y aumentando con la coexistencia de la misma. En el momento de la publicación de estos datos, no existían estudios que cuantificaran el estado del VV de la adventicia de un segmento arterial libre de placa de ateroma en

voluntarios sanos y en pacientes diabéticos. Sin embargo, hace más de cuatro décadas en un estudio post-mortem, Angervall et al[71] ya encontraron que los VV de la arteria aorta de pacientes diabéticos jóvenes tratados con insulina, mostraban los rasgos característicos de la microangiopatía diabética.

Recientemente, se han confirmado estos resultados en un estudio de otros investigadores en un grupo más amplio en pacientes con DM tipo 2[72]. Nuestro grupo de investigación ha estudiado la presencia de VV carotídeo en pacientes con DM tipo 1[73], con el objetivo de confirmar estos resultados en este tipo de diabetes, que representa una población con hiperglucemia y con menor carga de otros factores de riesgo cardiovascular que contribuyeran a la patogénesis de la arteriosclerosis. El análisis de datos de estos pacientes, ha mostrado también un aumento de la angiogénesis de los VV de la arteria carótida, apoyando la hipótesis de partida de nuestro estudio.

Con nuestros resultados, no encontramos asociación entre la señal de VV y las otras variables hematológicas, ni entre el señal de VV y la presencia de placas arterioscleróticas; es decir, la presencia de neovascularización intraplaca no mostró diferencias significativas entre pacientes con y sin RD. Sin embargo, el número de placas no era muy elevado, y el estudio de la neovascularización de las mismas requeriría otro estudio con un diseño específico.

Teniendo en cuenta estos datos, proponemos que la microcirculación de la pared de las grandes arterias es un tejido diana específico de la microangiopatía diabética, similar al observado clásicamente en la retina, el glomérulo renal y el nervio periférico. De esta forma, consideramos que existen otros posibles territorios afectos por las complicaciones microvasculares de la DM. Esto podría explicar la participación de la microangiopatía de los VV de la pared vascular en la patogénesis de la arterioscleorosis acelerada que observamos en pacientes diabéticos, y establecería un mecanismo fisiopatológico común que conectaría las complicaciones micro y macrovasculares diabéticas. Muy probablemente, la isquemia de la pared arterial estimularía la angiogénesis de los VV de la adventicia de la misma forma que la isquemia retininana lo haría en las formas más avanzadas y graves de RD.

La determinación del GIM y de la carga arteriosclerótica (presencia y número de placas de ateroma) en las arterias carótidas de nuestra población de estudio, demostró que los pacientes con DM tipo 2 con RD presentan una mayor prevalencia de arteriosclerosis subclínica en comparación con los pacientes sin RD. La inclusión de pacientes sin enfermedad cardiovascular acompañante y con función renal normal, permitió excluir otros factores de riesgo independiente de enfermedad arteriosclerótica. La RD se asoció de forma independiente con la presencia de placas carotídeas. No se observaron diferencias en la media global GIM de los tres territorios (carótida común, bulbo y carótida interna) entre los pacientes con y sin RD, excepto en la media y máxima de GIM de la carótida

común de los pacientes con RD. Tampoco se observaron diferencias en las mediciones de GIM o en la presencia de placa entre los grupos con diferentes grados de RD. Estudios recientes sugieren que las mediciones de placa carotídea son un predictor más fuerte de eventos CV que las mediciones del GIM[51,52]. Esto se explicaría por qué la hipertrofia de la media o el engrosamiento de la íntima en la arteria carótida común, pueden estar influenciados por factores que no reflejen necesariamente el proceso arteriosclerótico, como la edad y la hipertensión[74].

La presencia de mayor enfermedad ateromatosa subclínica carotídea en pacientes con RD podría contribuir a explicar los resultados de algunos estudios epidemiológicos que demuestran que la RD se asocia con una mayor morbilidad y mortalidad CV en pacientes con DM tipo 2[60]. En estos pacientes, la presencia de RD se ha descrito como un factor de riesgo independiente para la enfermedad coronaria incidente[61,62] y el accidente cerebrovascular isquémico[63].

7. CONCLUSIONES

- La baja capacidad de transporte de oxígeno en la sangre, medida como concentración de hemoglobina circulante, se asocia a estadios más avanzados de retinopatía diabética y a la presencia de isquemia retiniana. Esto nos permite proponer que la hipoxia relativa asociada a menor concentración de hemoglobina es un potencial factor contribuyente para el desarrollo y/o progresión de la retinopatía diabética, y para la aparición de isquemia retiniana.

- La baja capacidad transportadora de oxígeno de la sangre se asocia con cambios neuroretinianos precoces, evidenciados por una pérdida de grosor de capa de fibras nerviosas de la retina en pacientes sin retinopatía diabética y con retinopatía incipiente. El aumento de grosor y volumen macular en pacientes con diabetes tipo 2 con retinopatía diabética leve es un signo precoz que puede indicar el aumento de la permeabilidad vascular.

- Los pacientes con diabetes mellitus tipo 2, y de forma más acentuada los pacientes con retinopatía diabética, muestran un aumento de la angiogénesis de los vasa vasorum de la arteria carótida. Esto nos permite proponer que la microcirculación de la adventicia arterial es un tejido diana no clásico de la microangiopatía en la diabetes mellitus tipo 2. Esta microangiopatía de la pared arterial contribuiría, al menos en parte, al desarrollo y progresión de la arteriosclerosis en la diabetes. Esto ayuda a explicar la interconexión entre las complicaciones micro y macrovasculares presentes en estos pacientes.

- Los pacientes con retinopatía diabética presentan mayor frecuencia de enfermedad ateromatosa subclínica carotídea, medida como presencia y número de placas de ateroma, en comparación con los pacientes sin retinopatía, independientemente de otros factores que pueden contribuir a la arteriosclerosis.

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